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Chitosan and its quaternized derivative as effective long dsRNA carriers targeting shrimp virus in *Spodoptera frugiperda* 9 cells

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ABSTRACT

RNA interference (RNAi) is a promising strategy to combat shrimp viral pathogens at lab-scale experiments. Development of effective orally delivered agents for double-stranded (ds)RNA is necessary for RNAi application at farm level. Since continuous shrimp cell lines have not been established, we are developing a dsRNA-delivery system in *Spodoptera frugiperda* (Sf9) cells for studying *in vitro* RNAi-mediated gene silencing of shrimp virus. Sf9 cells challenged with yellow head virus (YHV) were used for validating nanoparticles as effective dsRNA carriers. Inexpensive and biodegradable polymers, chitosan and its quarternized derivative (QCH4), were formulated with long dsRNA (>100 bp) targeting YHV. Their morphology and physicochemical properties were examined. When treated with chitosan– and QCH4–dsRNA complexes, at least 50% reduction in YHV infection in Sf9 cells relative to the untreated control was evident at 24 h post infection with low cytoxicity. Inhibitory effects of chitosan– and QCH4–dsRNA complexes were comparable to that of dsRNA formulated with Cellfectin®, a commercial lipid-based transfection reagent. The natural and quaternized chitosan prepared in this study can be used for shrimp virus-specific dsRNA delivery in insect cultures, and have potential for future development of dsRNA carriers in shrimp feed.

1. Introduction

Chitosan and its derivatives as non-viral vectors have gained tremendous interest for their applications, especially for gene transfer and RNAi delivery systems. Chitosan can encapsulate nucleic acids, both DNA and RNA, and form nanopolyplexes *via* ionic interactions. The nanopolyplexes are low toxicity, biodegradable and biocompatibility (De Smedt et al., 2000; Koping-Hoggard et al., 2001). Chemical modifications and the ratio of positively charged chitosan and negatively charged nucleic acids are introduced to optimize stability of the nanopolyplex. Several previous studies demonstrated the capacity of chitosan or chitosan derivative to carry and deliver plasmid DNA and RNA both *in vivo* and *in vitro* systems (Koping-Hoggard et al., 2001; Rojanarata et al., 2008; Techaarpornkul et al., 2010; Weecharangsan et al., 2008). However, applications of chitosan are still limited due to its insolubility in neutral and basic pH. Moreover, its low specificity and transfection efficiency of chitosan should be overcome for its use in clinical trials (Kim et al., 2007). Quaternized chitosan (QCH) was developed to improve solubility of natural chitosan at pH ≥ 7. It was prepared by using commercially available Quat 188 under basic condition (Sajomsang et al., 2009a,b). The Quat 188 is an aqueous solution of *N*-(3-chloro-2-hydroxypropyl) trimethylammonium chloride (Xiao et al., 2012). It is well-known as a quaternizing agent that introduce quaternary ammonium moiety into the polymer backbone such as the one in starch (Heinze et al., 2004), cellulose (Hashem et al., 2003), and chitosan (Sajomsang et al., 2009a,b).

RNAi-based preventive approaches have shown promise against shrimp viruses at lab-scale experiments. To date, intramuscularly injection of dsRNA appears to be the most effective delivery method for RNAi-mediated shrimp viral inhibition (Ongvarrasopone et al., 2008; Saksmerprome et al., 2009; Tirasophon et al., 2007). There remains a need to improve oral delivery method to make RNAi-mediated antiviral strategy feasible for shrimp farming. Due to the lack of availability of continuous shrimp cell line, mosquito and *Spodoptera frugiperda* 9 (Sf9) cell cultures have gained interest for those who study shrimp-virus responses in molecular detail. They have been reported to be immunopositive to white spot syndrome virus (WSSV) and yellow head virus (YHV), suggesting likelihood of shrimp viruses persistently replicating in insect cells (Sriton et al.,

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2009; Gangnonngiw et al., 2010). The insect cells could be useful not only for studying RNAi-mediated gene silencing of shrimp virus but also for validating dsRNA carriers with promising inhibitory effects prior to testing in shrimp.

In this study, chitosan and its quarternized derivatives are prepared as previously described (Sajomsang et al., 2009a,b), and formulated with bacterially expressed dsRNA (>100 bp) targeting YHV RNA-dependent RNA polymerase (RdRp) gene. Complex formations were analyzed by physicochemical methods. The aim of this study is to evaluate transfection efficacy and cell toxicity of the chitosan–viral specific dsRNA complexes in an insect cell system. The effective chitosan nanoparticles determined from this study would have further applications in development of shrimp feed containing viral specific dsRNA.

2. Materials and methods

2.1. Preparation of chitosan and QCH4

The chitosan, with average molecular weights (M_w) of 276 was purchased from Seafresh Chitosan (lab) Co., Ltd. in Thailand. The degree of deacetylation (DDA) of chitosan was determined to be 94% by ^{1}H NMR spectroscopy (Lavertu et al., 2003). *N*-(3-Chloro-2-hydroxypropyl) trimethylammonium chloride (Quat 188) was obtained from the Dow Chemical Company in Thailand. A dialysis tubing with M_w cut-off of 12,000–14,000 g/mol from Cellu Sep T4, Membrane Filtration Products, Inc., (Segiun, TX, USA) were used to purify chitosan derivative. Chitosan was dispersed in 1% (w/v) acetic acid to prepare the stock solution at a final concentration of 1 μg/μl. QCH4 was synthesized by quaternizing chitosan with *N*-(3-chloro-2-hydroxypropyl) trimethylammonium chloride (Quat 188) (Sajomsang et al., 2009a,b).

2.2. Characterization of chitosan and QCH4

All attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra were collected with a Nicolet 6700 spectrometer (Thermo Company, USA) using the single-bounce ATR-FTIR spectroscopy (Smart Orbit accessory) with a diamond internal reflection element (IRE) at the ambient temperature (25 °C). These spectra were collected by using rapid-scan software in OMNIC 7.0 with 32 scans and a resolution of 4 cm^{-1}. The ^{1}H NMR spectra were measured on AVANCE AV 500 MHz spectrometer (Bruker, Switzerland). All measurements were performed at 300 K, using the pulse accumulation of 64 scans and LB parameter of 0.30 Hz. D_2O/CD_3COOD and D_2O were used as the solvents for dissolving 5 mg of chitosan and QCH4, respectively. The weight average molecular weight (M_w), number average molecular weight (M_n), and M_w/M_n of chitosan and QCH4 were determined by using the gel permeation chromatography (GPC). It consists of Waters 600E Series generic pump, injector, ultrahydrogel linear columns (M_w resolving range 1–20,000 kDa), guard column, pollulans as standard (M_w 5.9–788 kDa), and refractive index detector (RI). All samples were dissolved in acetate buffer pH 4 and then filtered through VertiPure nylon syringes filters 0.45 μm (Vertical chromatography Co., Ltd., Thailand). The mobile phases, 0.5 M AcOH and 0.5 M AcONa (acetate buffer pH 4), were used at a flow rate of 0.6 ml/min at 30 °C. Then the injection volume 20 μl was used.

2.3. Formation of chitosan–dsRNA and QCH4–dsRNA nanopolyplexes

Using the recombinant plasmid with YHV RdRp hairpin gene, dsRNA was prepared in *E. coli* HT115 (DE3) as previously described (Saksmerprome et al., 2009). One microgram of chitosan and QCH4 solution were mixed with 1 μg of dsRNA solution. The complexes were formed through a self-assembly mechanism after pipetting, and subsequently incubated at room temperature for 15 min before use. Different weight ratios of chitosan–dsRNA and QCH4–dsRNA complex were prepared to investigate dsRNA binding affinity by gel retardation. Physicochemical characterization and morphological analysis of the obtained nanoplexes were then examined. Hydrodynamic diameter, polydispersity index (PDI) and zeta potential of nanopolyplexes were determined by the Photon Correlation Spectroscopy machine and electrophoretic mobility titration (NanoZS4700 nanoseries, Malvern Instruments, UK). The samples were obtained as the average of three measurements at 25 °C. For morphology investigation, 5 μl of each of dsRNA, chitosan, QCH4, chitosan–dsRNA complex and QCH4–dsRNA complex were placed on a freshly cleaved mica surface, dried with a stream of nitrogen, and further dried in an electronic dry cabinet at 25 °C for 30 min. All samples were determined by atomic force microscope (SPA400, Seiko, Japan) machine used the scanner range 10–1000 nm area in tapping mode using a Micro cantilever with 28 kHz resonance frequencies and a constant force, range of 1.4–1.8 N/m. All images were recorded in air at room temperature and a scan speed of 0.8 Hz and the phase image and topology used to determine the morphology and particle size.

2.4. Transfection of dsRNA into Sf9 cells using Cellfectin® (Invitrogen, USA) and chitosan nanopolyplexes

Sf9 cells were cultured in 2 ml of Sf-900 III SFM containing 1% antibiotic-antimycotic (pH 6.2) at a concentration of 5×10^5cells/ml for 6-well plate (Costar, Corning, USA) and incubated at 28 °C overnight or until 60–70% cell confluency. Using 5 μl Cellfectin®, 2 μg of dsRNA were transfected into Sf9 cells by Cellfectin® (Gibco Invitrogen, USA). The treated Sf9 cells were incubated at 28 °C for 12 h prior YHV infection. YHV stock was prepared from hemolymph of YHV-infected shrimps as previously described (Saksmerprome et al., 2009). Sf9 cells were infected with YHV at a multiplicity of infection (MOI) of 20 or around 2×10^7 copies/well by incubating on a shaker at 28 °C for 6 h. In the case of chitosan–dsRNA complex, 10 μl of complex which contains 2 μg of dsRNA was diluted in 1 ml Sf-900 III SFM without antibiotics prior to transfection.

Cells were harvested at 12 h after transfection by centrifugation at 2200 × *g* for 5 min. Total RNAs were extracted by Tri-pure reagent (Roche, USA). RNA concentrations were determined by UV spectroscopy at OD_{260}. First strand cDNA was prepared from 150 ng/μl of total RNA using Titan one tube reverse transcriptase (Roche, USA) at 50 °C for 30 min. Double-stranded RNA product in each RNA sample was monitored by specific primer YHV_invert_F_sal (5′-ACGCGTCGACGCATGTCCTGTTCTC-3′) and YHV_reverse_R_pst (5′-TTGACGTCGAATTCTAGCCATGC-3′). Actin-specific primer set, Actin-Sf9-Forward (5′-GATATGGAGAAGATCTGGCAC-3′) and Actin-Sf9-Reverse (5′-ACGGGTCTGTTCCCTATGAAGCACCAC-3′), was added in PCR reaction for internal control. PCR conditions were as followed: the initial denaturing step at 94 °C, 2 min; denaturing 94 °C, 30 s, annealing temperature depending on primers, 30 s, extension at 72 °C, 30 s for 30 cycles; and a final extension at 72 °C, 5 min. PCR products were analyzed using 1.5% agarose gel electrophoresis.

2.5. Preparation of the FITC-conjugated QCH4 and transfection

Fifty milligrams of QCH4 (0.3 mmol) were dissolved in deionized water (5 ml). Twelve milligram of fluorescein isothiocyanate (FITC, 0.1 equiv./GlcN) was dissolved dimethylsulfoxide (DMSO, 3 ml) and adjusted to pH of 3.0 with 1 M HCl. Six milligram of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 0.1 equiv./GlcN) was