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Abstract

Objective The feasibility of chitosan as an alternative transfection reagent candidate was examined for protein expression in Bm5 cells and silkworm larvae using recombinant BmNPV bacmid DNA.

Results Chitosan 100 and recombinant *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid DNA, in amino group/phosphate group (N/P) ratios of 0.1–10, were used for formation of chitosan/DNA nanocomplexes. The chitosan/BmNPV bacmid DNA nanocomplexes showed higher specific activity of GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase 2 (β3GnT2) fusion protein (GGT2) expressed in silkworm larvae than DMRIE-C, a conventional silkworm transfection reagent. In particular, the composition of chitosan and BmNPV bacmid DNA nanocomplexes formed by an N/P ratio of 8 or 10, respectively, showed the highest specific activity of β3GnT2 in the silkworm larvae hemolymph. In addition, 3 different proteins were expressed in silkworm larvae to the same extent using chitosan as that using DMRIE-C.

Conclusion This is the first finding that chitosan/BmNPV bacmid DNA nanocomplexes can rival the performance of commercially available transfection reagents for the expression of recombinant proteins in Bm5 cells and silkworm larvae.

Keywords Chitosan · Silkworm · BmNPV bacmid · Recombinant protein ·Nanocomplex · β1,3-*N*-Acetylglucosaminyltransferase · Chitosan-DNA nanocomplexes

Introduction

Chitosan is a cationic, water-soluble, linear polymer extracted from crustacean shells that easily forms nanocomplexes with drugs, nucleic acids and proteins through electrostatic interactions. This interaction between chitosan and biomolecules leads to the production of biomaterials for a variety of biomedical applications, such as drug and gene delivery and tissue engineering (Garcia-Fuentes and Alonso, 2012). The biocompatibility and biodegradability properties of chitosan make it suitable for most in vivo medical applications. In particular, chitosan can be digested by the lysozymes *N*-acetylglucosaminidase and chitotriosidase, which exist in human mucosa and various physiological fluids (Gorzelanny et al., 2010; Kean et al., 2010).

Chitosan also can be utilized for gene delivery as a non-viral delivery system instead of cationic polymers and liposomes. Chitosan has protonated amine groups, the positive charge of which can electrostatically interact with DNA and RNA, allowing chitosan to be delivered into cells as nanocomplexes. Chitosan-nucleic acid nanocomplexes can be prepared by coacervation at low cost. In several reports, chitosan was modified by thiamine pyrophosphate, polyethylenimine and hyaluronic acid to enhance its in vitro transfection efficiency compared with that of commercially available transfection reagents (de la Fuente et al., 2008; Jian et al., 2009; Rojanarata et al., 2008; Steg et al., 2011; Tripathi et al., 2012). In the transfection of nucleic acids to cultured cells and tissues using chitosan, plasmid vectors, siRNA and shRNA have normally been used to express recombinant proteins and suppress the expression of specific genes.

In this study, chitosan was used instead of commercially available transfection reagents for recombinant protein expression in *Bombyx mori* ovary (Bm5) cells and silkworm larvae. To express recombinant proteins in Bm5 cells and silkworm larvae,

recombinant *B. mori* nucleopolyhedrovirus (BmNPV) bacmid DNA was also used (Motohashi et al., 2005). The size of the BmNPV bacmid is approximately 130 kbp, which is much larger than that of plasmid vectors; the transfection of large DNA such as the BmNPV bacmid has not previously been conducted. To confirm the transfection efficiency, GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase 2 (β3GnT2) fusion protein (GGT2) was expressed in Bm5 cells and silkworm larvae (Park et al., 2007) using the chitosan/BmNPV bacmid DNA nanocomplexes. To demonstrate the versatility of these chitosan-based nanocomplexes, rat α2,6-sialyltransferase (ST6), hemagglutinin (HA) from an influenza A virus, and the *Neospora caninum* surface protein (NcSRS2) were expressed using the chitosan/BmNPV bacmid DNA nanocomplexes. The efficiency and cost of this expression method compared with a commercial transfection reagent was also discussed.

Materials and methods

Construction of recombinant BmNPV bacmids

Recombinant BmNPV bacmids containing the GGT2 gene (Kato et al., 2004), recombinant BmNPV bacmids for the expression of rat ST6 (Ogata et al., 2009) and NcSRS2 (Otsuki et al. 2013) have been constructed in previous studies. The construction of recombinant BmNPV bacmids containing the HA gene from an influenza A virus is briefly described below. A plasmid vector containing the HA gene from influenza A H5N8 (A/duck/NY/191255-59/02) was purchased from Sino Biological Inc. (Beijing, China). The FLAG-tagged HA gene lacking the sequence coding its signal peptide was amplified by PCR using HA-1st-F

(5’-GACTACAAGGATGACGATGACAAGTGTATTGGCTACCATGCCAACAAC-3’) and HA-1st-R (5’-GGGGTACCTTAGATACAAATCCTACATTGGAGGGA-3’). Using the amplified HA gene as a template and primers (HA-2nd-F: 5’-CGGGATCCATGGAGAAGATTGTGCTGCTGCTGGCTATTGTGTCCCTGGTGAAGTCTGACCAGATTGACTACAAGGATGACG-3’, HA-1st-R), the FLAG-tagged whole HA gene was amplified by PCR. The amplified, FLAG-tagged whole HA gene was inserted into the pFastBac1 vector and the constructed recombinant vector was transformed into *Escherichia coli* BmDH10bac $CP^{-}Chi^{-}$ (Park et al., 2008). Recombinant BmNPV bacmids containing the HA gene were extracted from a white colony through blue-white selection by the PureLink HiPure Plasmid DNA Purification Kit (Life Technologies Japan, Tokyo, Japan)

Preparation of the chitosan/BmNPV bacmid DNA nanocomplexes

Chitosan 10, 100, and 500 were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Chitosan was dissolved with 50 mM acetate, and the pH was adjusted to 5.5 using 0.1 M NaOH. Recombinant BmNPV bacmid DNA was dissolved with 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.5). In brief, 0.1% (w/v) Chitosan and each amount of recombinant BmNPV bacmid DNA were prepared with MES buffer (pH 6.5), heated separately to 50–55°C, and quickly mixed with each other according to amino group/phosphate group (N/P) ratio. The N/P ratios were calculated by the formula described below.

$$\text{N/P ratio} = \frac{Chitosan\ (g) \times 0.8}{DNA\ (g)} \times \frac{330\ (average\ molecular\ weight\ of\ each\ nucleotide)}{161\ (molecular\ weight\ of\ deacetylated\ GlcNAc)}$$

The chitosan 100 used in this study was approximately 80% deacetylated chitin. This mixture was incubated for 1 h at room temperature and subsequently used as the chitosan/BmNPV bacmid DNA nanocomplexes. Size and zeta potential of these nanocomplexes were analyzed by dynamic light scattering (DLS) analysis (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK).

Cultivation of Bm5 cells and rearing silkworm larvae

The Bm5 cells were cultivated at 27°C in Sf-900II medium (Life Technologies Japan) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich Japan, Tokyo, Japan) and 100-fold-diluted Antibiotic-Antimycotic (Life technologies Japan). Fourth instar silkworms were purchased from Ehime Sansyu (Ehime, Japan) and reared at 26°C on an artificial diet (Silkmate 2S, Nosan, Yokohama, Japan).

Agarose gel electrophoresis analysis of chitosan/BmNPV bacmid DNA nanocomplexes

Chitosan/BmNPV bacmid DNA nanocomplexes containing 10 μg of BmNPV bacmid DNA were treated with 2 U of DNase (RT-grade, Wako) for 15 min at 37°C, and the reaction was stopped by adding the stop solution provided. The reaction mixture was analyzed using 1% agarose gel electrophoresis.

Transfection and injection of chitosan/BmNPV bacmid DNA nanocomplexes into Bm5 cells and silkworm larvae

Using a 6-well plate, 8×10^5 Bm5 cells were cultivated in each well. After removal of the culture medium, Cellfectin II (Life Technologies Japan)-BmNPV bacmid DNA

complexes or chitosan/BmNPV bacmid DNA nanocomplexes in Sf-900 II (BmNPV bacmid DNA: 2.5, 5 and 10 μg) were added into each well, and the plates were incubated for 5 h. Two milliliter of fresh culture medium supplemented with 10% FBS was added into each well following the removal of the Cellfectin II-BmNPV bacmid DNA complexes. Transfected Bm5 cells were cultivated for 7 d. In the case of chitosan/BmNPV bacmid DNA nanocomplexes, 2 ml of fresh culture medium supplemented with 10% FBS was added into each well, the nanocomplexes were not removed, and the cells were cultivated for 7 d. Cells and culture supernatants were collected and used for further experiments.

DMRIE-C (a 1:1 (M/M) liposome formulation of the cationic lipid DMRIE (1,2-dimyristyloxy-propyl-3-dimethyl-hydroxy ethyl ammonium bromide) and cholesterol in membrane filtered water, Life Technologies Japan)-BmNPV bacmid DNA complexes or chitosan/BmNPV bacmid DNA nanocomplexes (BmNPV bacmid DNA: 5 μg) were injected into fifth instar silkworm larvae, and the injected larvae were reared for 7 d on an artificial diet (Silkmate S2, Nohsan Corporation, Yokohama, Japan). Hemolymph and body fat were collected from the injected silkworm larvae and used for further experiments.

Culture supernatants and hemolymph were centrifuged at 10,000 × g, and the resulting supernatant was used as a sample. Cells and body fat were suspended with PBS (pH 7.4) and disrupted by sonication. The homogenate was centrifuged at 10,000 × g, and its supernatant was used as a sample.

β3GnT assay and protein concentration measurement

The β3GnT assay was conducted according to a previously reported method (Kato et al., 2004). The protein concentration of the silkworm hemolymph was determined using a BCA protein assay kit (Thermo Fisher Scientific K.K., Yokohama, Japan).

Detection of expressed proteins by fluorescence microscopy, SDS-PAGE and western blot

Transfected Bm5 cells were immobilized on a glass slide coated with aminosilane (Matsunami Glass, Osaka, Japan). Cells were fixed with 10% formalin and washed by PBS 4 times. Fluorescence was observed using a confocal laser scanning microscope (LSM700, Carl Zeiss Japan, Tokyo, Japan).

Expression of recombinant proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% or 12% acrylamide gels. In the case of GGT2, the samples were mixed with sample buffer (Aoki et al., 1996) without boiling and processed with SDS-PAGE. GGT2 was detected using Molecular Imager FX (Bio-Rad, Hercules, CA, USA). Other recombinant proteins were detected by western blot. After SDS-PAGE, proteins were electrotransferred onto a polyvinylidene fluoride (PVDF) membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). After blocking in 5% skimmed milk in TBS containing 0.1% Tween 20 (TBST), the membrane was incubated for 1 h in 1:10000 mouse anti-DDDDK antibody (Medical & Biological Laboratories, Nagoya, Japan). The membrane was washed with TBST and incubated for 1 h in 1:20000 anti-mouse IgG antibody labeled with horseradish peroxidase (GE Healthcare Japan, Tokyo, Japan). Detection was performed with Immobilon Western Chemiluminescent HRP Substrate

(ECL) (Merck Millipore, Billerica, MA, USA). Specific protein bands were detected using a Fluor-S MAX MultiImager (Bio-Rad).

Results

Preparation of the chitosan/BmNPV bacmid DNA nanocomplexes

Chitosan is cationic because of its amine groups. In this study, the ratio of acetylated amine groups had a great effect on chitosan solubility. The chitosan 10, 100 and 500 used were deacetylated by more than 80%, according to the commercial information. In a preliminary experiment, pH 6.5 MES buffer was shown to be favorable for forming chitosan/BmNPV bacmid DNA nanocomplexes using Chitosan 100. Optimal pH for the chitosan/DNA nanocomplexes to obtain the adequate balance between DNA association and dissociation is slightly lower than 7 (Mao et al., 2010). Therefore, we used pH 6.5 MES buffer for the preparation of the chitosan/BmNPV bacmid DNA nanocomplexes.

Chitosan 100 was used to prepare the chitosan/BmNPV bacmid DNA nanocomplexes. Different types of these nanocomplexes were prepared using various N/P ratios ranging from 0.1–10; their resulting sizes and zeta potentials are shown in Fig. 1. As the N/P ratio increased, the nanocomplexes increased in size from approximately 70 nm to 120 nm. When the chitosan formed nanocomplexes with sodium sulfate in the absence of DNA they were approximately 80 nm in diameter. Fig. 1 indicates that at N/P ratios greater than 2, Chitosan 100 can form larger nanocomplexes with BmNPV bacmid DNA than that without DNA. In a previous study,

large aggregates were found when chitosan and plasmid DNA were mixed at an N/P ratio of approximately 1 (Mao et al., 2001), but no aggregation was detected in this study; these nanocomplexes were smaller than 130 nm for all N/P ratios (Fig. 1). Chitosan/BmNPV bacmid DNA nanocomplexes prepared at N/P ratios ranging from 0.1–10 were analyzed using agarose gel electrophoresis (Fig. 2a). The size of BmNPV bacmid DNA is approximately 130 kbp, and it is observed above 10 kbp in an agarose gel. At N/P ratios of 0.1 and 0.3, some of the BmNPV bacmid DNA was observed around the wells and the rest was observed at the same location as naked BmNPV bacmid DNA, indicating that chitosan/BmNPV bacmid DNA partially formed at these N/P ratios. At N/P ratios greater than 0.5, all of the BmNPV bacmid DNA formed nanocomplexes with chitosan. Next, DNase treatment of chitosan/BmNPV bacmid DNA was performed to confirm the protection of the BmNPV bacmid DNA by chitosan in these nanocomplexes (Fig. 2b). BmNPV bacmid DNA was not observed in the nanocomplexes at N/P ratios of 0.5, 0.8, and 1 after DNase treatment, but it was observed in nanocomplexes at N/P ratios greater than 4 even though DNase treatment was performed. This result indicates that chitosan/BmNPV bacmid DNA nanocomplexes formed incompletely between N/P ratios of 0.5 and 1 because the amount of chitosan was limited; at N/P ratio greater than 4, nanocomplexes completely coated with chitosan formed, which completely protected BmNPV bacmid DNA from digestion by DNase.

Expression of recombinant proteins in Bm5 cells using chitosan/BmNPV bacmid DNA nanocomplexes

Chitosan/BmNPV bacmid DNA nanocomplexes were prepared at N/P ratios of 4 and 6 using chitosan 100 and BmNPV bacmid DNAs containing the GGT2 fusion gene, and these nanocomplexes were transfected into Bm5 cells. Green fluorescence was not only observed in Bm5 cells transfected with Cellfectin II-BmNPV bacmid DNA complexes but also in Bm5 cells transfected with chitosan/BmNPV bacmid DNA nanocomplexes (Fig. 3a). However, Bm5 cell transfection only with bacmid DNA did not yield any green fluorescence. These results indicate that chitosan 100 can function at the same level as Cellfectin II as a Bm5 cell transfection reagent. GGT2 expression in Bm5 cell was used to assess BmNPV bacmid DNA doses between 2.5 and 10 μg at N/P ratios 4 and 6. SDS-PAGE analysis of intracellular GGT2 expression was higher using nanocomplexes at an N/P ratio of 6 than that at an N/P ratio of 4 (Fig. 3b). In all conditions at an N/P ratio of 6, 5 μg of BmNPV bacmid DNA in each well of a 6-well plate (8×10^5 cells) yielded the highest expression of GGT2 (Fig. 3b). These results indicate that chitosan 100 can be used for protein expression using BmNPV bacmid in Bm5 cells as a transfection reagent instead of Cellfectin II.

Expression of recombinant proteins in silkworm larvae using chitosan/BmNPV bacmid DNA nanocomplexes

GGT2 in silkworm larvae was expressed using the chitosan/BmNPV bacmid DNA nanocomplexes. DMRIE-C was used as a transfection reagent for silkworm larvae and pupae. In the case of the DMRIE-C reagent, 8 out of 10 silkworm larvae exhibited green fluorescence at 7 d after injection of the DMRIE-C-BmNPV bacmid DNA complexes (Fig. 4a). Compared to DMRIE-C, almost the same transfection efficiency (5–8 out of 10 silkworm larvae) was achieved by injection of chitosan/BmNPV bacmid DNA

234 nanocomplexes between N/P ratios of 2 and 8 (Fig. 4a). Injection of only naked
235 recombinant BmNPV bacmid DNA did not allow GGT2 to be expressed in silkworm
236 larvae. GGT2 expression was observed in the hemolymph from silkworm larvae
237 injected with DMRIE-C-BmNPV bacmid DNA complexes, chitosan/BmNPV bacmid
238 DNA nanocomplexes between N/P ratios 2 and 8, and mock (Fig. 4b). Specific β3GnT
239 activity observed in the hemolymph at each N/P ratio was higher than that observed
240 after using DMRIE-C (Fig. 5). In particular, the silkworm larvae injected with
241 chitosan/BmNPV bacmid DNA nanocomplexes with N/P ratios of 8 or 10 exhibited the
242 highest specific activity of β3GnT in the hemolymph. These results indicate that
243 chitosan 100 can be also used for protein expression using BmNPV bacmid in silkworm
244 larvae as a transfection reagent instead of DMRIE-C.

245 Feasibility of chitosan/BmNPV bacmid DNA nanocomplexes as transfection agent for
246 expression of recombinant proteins in silkworm larvae

247 The expression of rat ST6, HA from the influenza A H5N8 virus and NcSRS2 was
248 investigated using chitosan/BmNPV bacmid DNA nanocomplexes in silkworm larvae.
249 Rat ST6 and NcSRS2 from *N. caninum* were expressed in the hemolymph because the
250 transmembrane domain of each protein was deleted (Ogata et al., 2009; Otsuki et al.,
251 2013). All three recombinant proteins were expressed in the silkworm larvae
252 hemolymph (Fig. 6). Expressed HA of the influenza A H5N8 virus was observed in the
253 hemolymph at approximately 50 kDa even when this HA had its own transmembrane
254 domain. This result suggested that the HA expressed in the hemolymph was observed as
255 HA1 because the estimated molecular weight of this HA in Fig. 6 is approximately 50

kDa. These results indicate that chitosan can be used as an inexpensive substituent for DMRIE-C for the expression of any recombinant protein in silkworms.

GGT2 was also expressed in silkworm larvae using chitosan 10 and 500 instead of chitosan 100. Chitosan 10 has the smallest molecular weight among these three types of chitosan, while Chitosan 500 has the largest. In addition, chitosan 10 and 500 mediated the expression of GGT2 in the hemolymph to the same extent as did chitosan 100 (data not shown).

Discussion

The transfection efficiency of DNA into cells by chitosan depends on various parameters, including the molecular weight and deacetylation degree of chitosan, the N/P ratio of chitosan/DNA particles, the pH, and additives, among others (Mao et al., 2010). In this study, at N/P ratios between 2 and 10, higher levels of GGT2 expression in hemolymph were observed than those resulting from use of DMRIE-C. In the BmNPV bacmid-silkworm expression system, BmNPV is produced and amplified, leading to a systemic infection in the silkworm larvae when even the slightest amount of BmNPV bacmid is introduced into silkworm larvae cells. This suggests that perfect transfection conditions are not needed for the recombinant protein expression in silkworm larvae. However, nanocomplexes composed of chitosan/BmNPV bacmid DNA at N/P ratios of 8 or 10, respectively, showed the highest specific activity of β3GnT in the silkworm larvae hemolymph. This result indicates that a high N/P ratio is favorable for the expression of recombinant proteins in the BmNPV bacmid-silkworm system. In general, higher molecular weight chitosan provides high transfection efficiency at lower N/P ratio, but lower molecular weight chitosan requires higher N/P

279 ratios to form chitosan/DNA nanocomplexes completely (Lavertu et al., 2006; Romøren
280 et al., 2003). To obtain high transfection efficiency with this chitosan-based formulation,
281 the optimization of N/P ratio and chitosan molecular weight is required (Mao et al.,
282 2010; Sato et al, 2001). The average molecular weight of the chitosan 100 used in this
283 study has not been informed from the manufacturer. However, from the result shown in
284 Fig. 4, it is possible that further optimization of the N/P ratio and chitosan molecular
285 weight will provide the highest expression of recombinant proteins in BmNPV
286 bacmid-silkworm system. In this case, it should be taken into consideration that the size
287 of BmNPV bacmid DNA is larger than that of plasmid DNA and siRNA, which have
288 been normally used for chitosan-based transfection.
289 In this study, recombinant proteins were expressed in Bm5 cells and silkworm
290 larvae using chitosan instead of the conventional transfection reagents Cellfectin II and
291 DMRIE-C. GGT2 expression in silkworm larvae hemolymph using chitosan 100 was
292 higher than that achieved by using DMRIE-C. Similar rat ST6 expression resulted from
293 the use of chitosan 100 and DMRIE-C. In addition, HA from the influenza A H5N8
294 virus and NcSRS2 from *N. caninum* exhibited comparable expression resulting from the
295 use of chitosan 100 and DMRIE-C. Conventionally, DMRIE-C, provided by Life
296 Technologies, is used to express recombinant proteins in silkworms by injecting
297 recombinant BmNPV bacmid DNA. In this case, 5 μl of DMRIE-C is needed to inject
298 recombinant bacmid DNA into a silkworm larva, and its cost for the recombinant
299 protein expression in a silkworm is 2.6 US$. Using chitosan 100, 36 μg of chitosan 100
300 is required for the recombinant protein expression in a silkworm, and its cost is $4.0 \times$
301 10^{-7} US$. Chitosan 100 is much less expensive to use than DMRIE-C for the expression

of recombinant proteins in silkworms and may lead to the cost-effective, large-scale production of recombinant proteins in silkworms.

In conclusion, we developed a highly cost-effective transfection method using chitosan 100. Chitosan 100 and recombinant BmNPV bacmid DNA easily formed chitosan/BmNPV bacmid DNA nanocomplexes, which protected DNA from digestion by DNase when prepared with an N/P ratio greater than 4 and an average size of 100 nm. Using these nanocomplexes as well as the commercial transfection reagent Cellfectin II, several proteins were expressed successfully. In the case of GGT2, the composition of chitosan and BmNPV bacmid DNA nanocomplexes formed by an N/P ratio of 8 or 10, respectively, showed the highest specific activity of β3GnT2 in the silkworm larvae hemolymph. In addition, recombinant proteins originating from a rat, influenza virus and *Neospora caninum* surface protein were expressed in the silkworm larvae using chitosan 100 to the same extent as expression resulting from the use of DMRIE-C. This is the first demonstration of chitosan/BmNPV bacmid DNA nanocomplexes rivaling silkworm protein expression achieved by commercially available transfection reagents, and these results may lead to significantly reduced cost for recombinant protein expression compared with the cost of conventional transfection methods.

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Figure legends

Fig. 1 Particle size and zeta potential of chitosan/BmNPV bacmid DNA nanocomplexes in the N/P ratio range of 0.1–10. These particles were prepared using Chitosan 100 and recombinant BmNPV bacmid harboring GGT2 gene at each N/P ratio. Particle size and zeta potential of these particles were analyzed dynamic light scattering (DLS) analysis.

Fig. 2 Electrophoretic mobility analysis of chitosan/BmNPV bacmid DNA nanocomplexes. **A** Agarose gel electrophoresis of chitosan/BmNPV bacmid DNA nanocomplexes of various N/P ratios of 0.1–10. **B** Agarose gel electrophoresis of DNase-treated chitosan/BmNPV bacmid DNA nanocomplexes of various N/P ratios of 0.5–10. (+) and (-) denote chitosan/BmNPV bacmid DNA nanocomplexes treated with DNase and without, respectively.

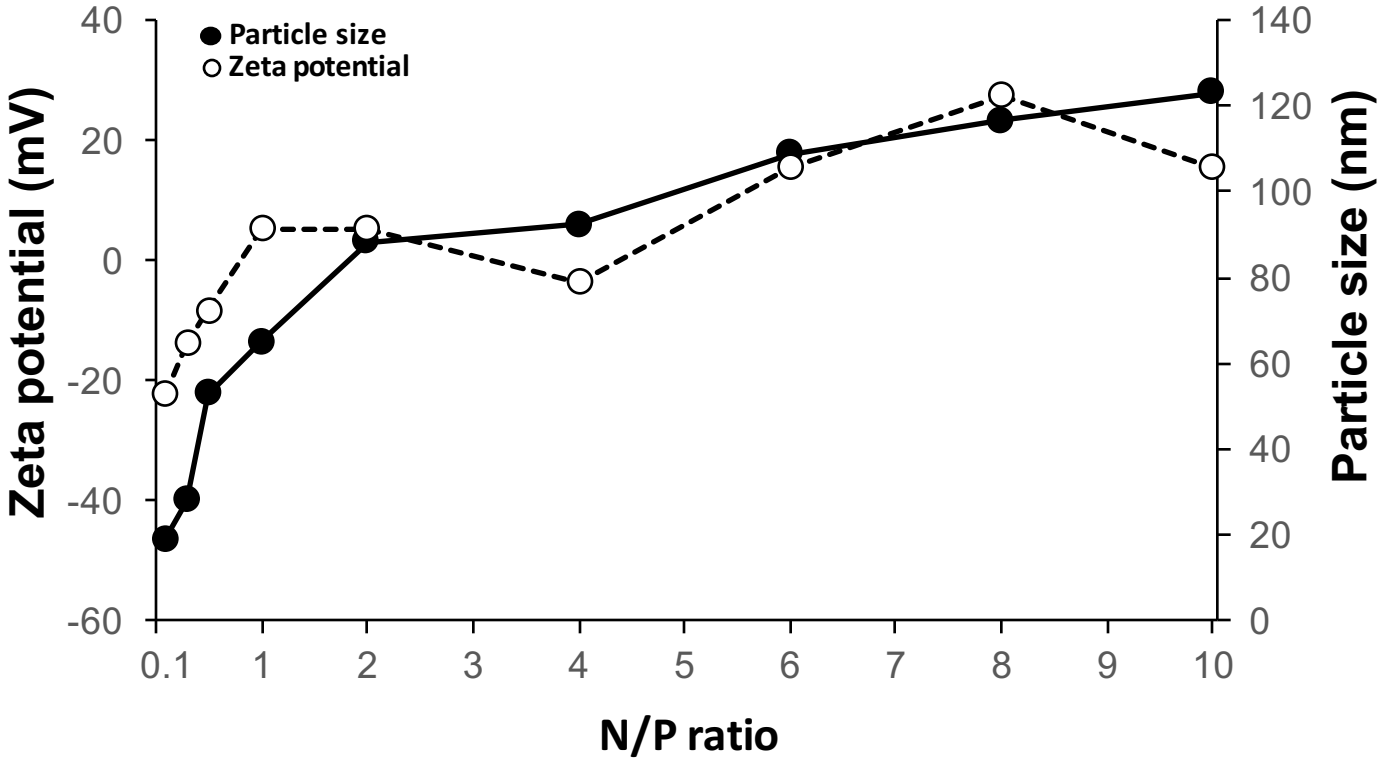
Fig. 3 Expression of GGT2 in Bm5 cells using chitosan/BmNPV bacmid DNA nanocomplexes. **A** Fluorescent microscopy of Bm5 cells transfected with chitosan/BmNPV bacmid DNA nanocomplexes. Chitosan 100 and recombinant BmNPV bacmid harboring GGT2 gene at each N/P ratio were used for chitosan/BmNPV bacmid DNA nanocomplexes. After 7 d incubation, green fluorescence in Bm5 cells was detected by confocal fluorescence microscope. **B** Expression of GGT2 in Bm5 cells transfected with chitosan/BmNPV bacmid DNA nanocomplexes prepared at N/P ration 4 or 6. Used amount of recombinant bacmid DNA was 2.5, 5, and 10 μg. Green fluorescent bands of GGT2 on an SDS-PAGE gel were detected by Molecular imager FX.

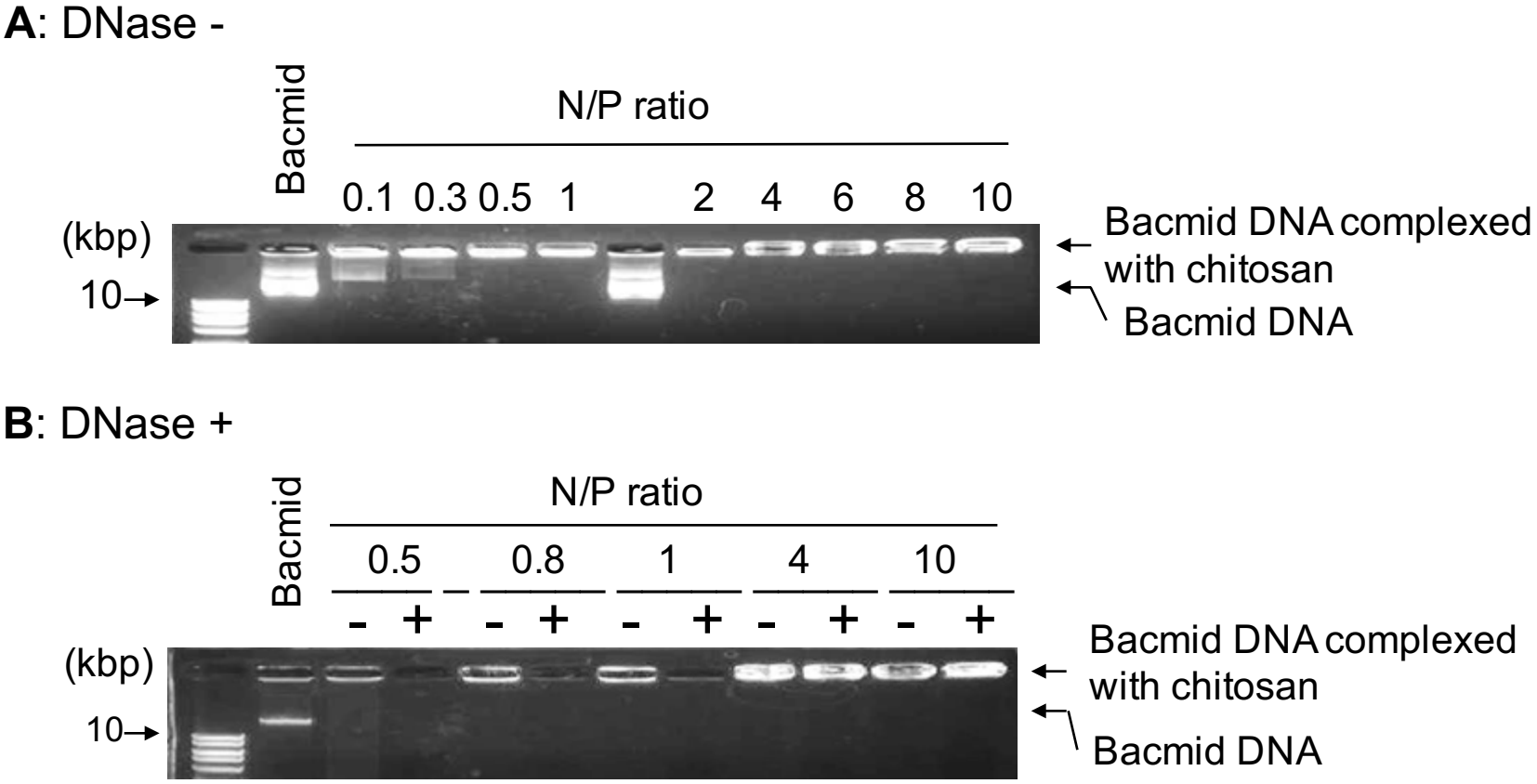
Fig. 4 Expression of GGT2 in silkworm larvae using chitosan/BmNPV bacmid DNA nanocomplexes. **A** Green fluorescent of DMRIE-C- and chitosan/BmNPV bacmid DNA nanocomplexes-injected 10 silkworm larvae with under UV light. **B** Expression of GGT2 in silkworm larvae injected with chitosan/BmNPV bacmid DNA nanocomplexes prepared at each N/P ration using 5 μg of recombinant bacmid DNA. Green fluorescent bands of GGT2 in hemolymph on an SDS-PAGE gel were detected by Molecular imager FX.

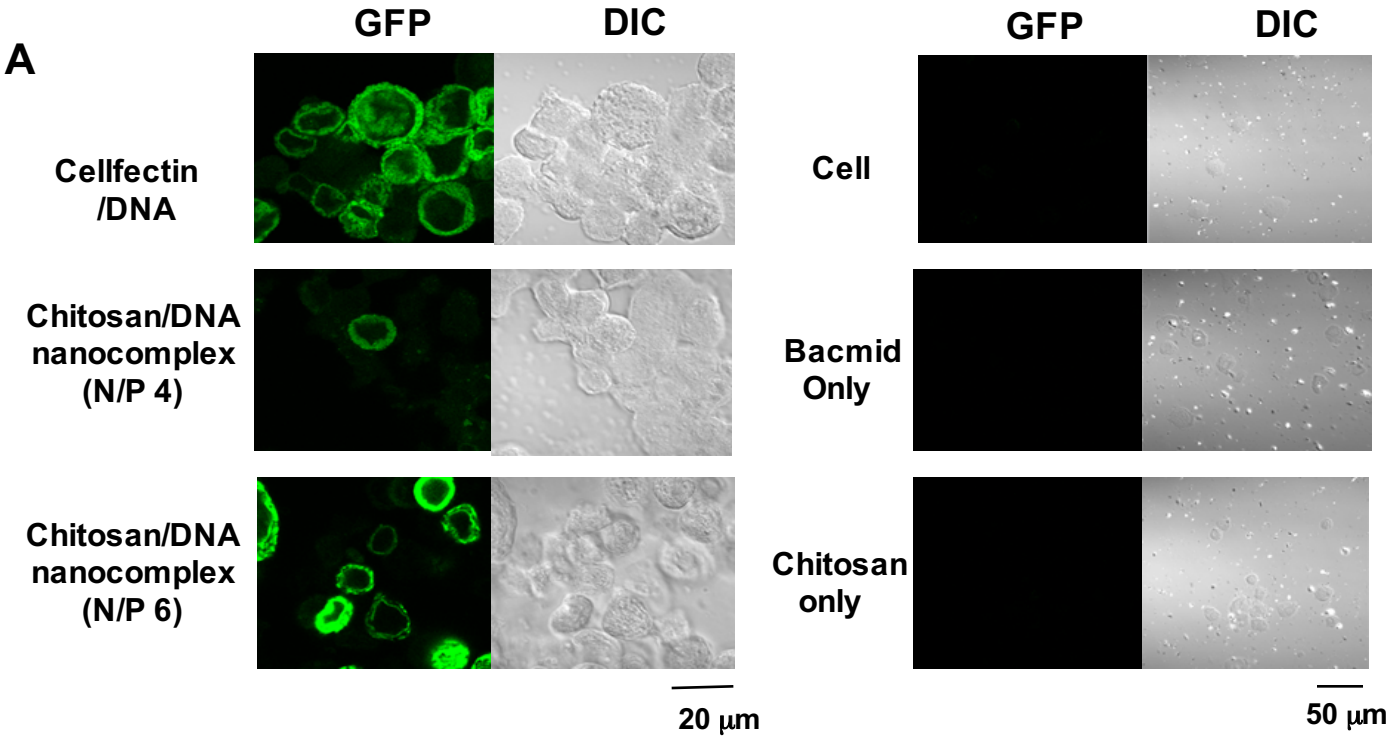
Fig. 5 Specific β3GnT activity of hemolymph in silkworm larvae using chitosan/BmNPV bacmid DNA nanocomplexes at each N/P ratio. Five microgram of recombinant BmNPV bacmid was used for protein expression in silkworm larva. β3GnT assay and protein concentration measurement are described in Materials and methods (n=3). Error bars indicate standard deviation. Student's t-test was performed to find significant difference between two means ($p<0.05$).

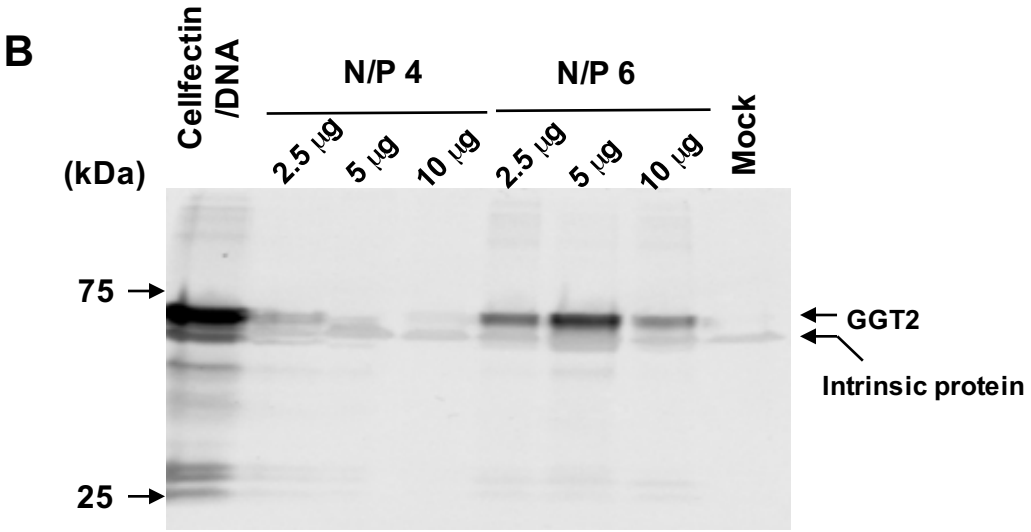
Fig. 6 Expression of rat ST6, HA from influenza A H5N8 virus and NcSRS2 from *N. caninum* in chitosan/BmNPV bacmid DNA nanocomplexes-injected silkworm larval hemolymph. Chitosan 100 and each recombinant bacmid DNA were used at N/P ratio 5 for the preparation of chitosan/bacmid DNA nanocomplexes. Five microgram of recombinant BmNPV bacmid was used for protein expression in silkworm larva. Lane1: Mock, lane 2: DMRIE-C-BmNPV bacmid DNA, lane 3: Chitosan/BmNPV bacmid DNA nanocomplex.

Fig. 1 Kato et al.




A: DNase -
Bacmid
N/P ratio
0.1 0.3 0.5 1 2 4 6 8 10
(kbp)
10
Bacmid DNA complexed with chitosan
Bacmid DNA
B: DNase +
Bacmid
N/P ratio
0.5 0.8 1 4 10
- + - + - + - + - +
(kbp)
10
Bacmid DNA complexed with chitosan
Bacmid DNA


A
GFP
DIC
GFP
DIC
Cellfectin /DNA
Cell
Chitosan/DNA nanocomplex (N/P 4)
Bacmid Only
Chitosan/DNA nanocomplex (N/P 6)
Chitosan only
20 μm
50 μm


B
Cellfectin /DNA
N/P 4
N/P 6
2.5 μg
5 μg
10 μg
2.5 μg
5 μg
10 μg
Mock
(kDa)
75
25
GGT2
Intrinsic protein

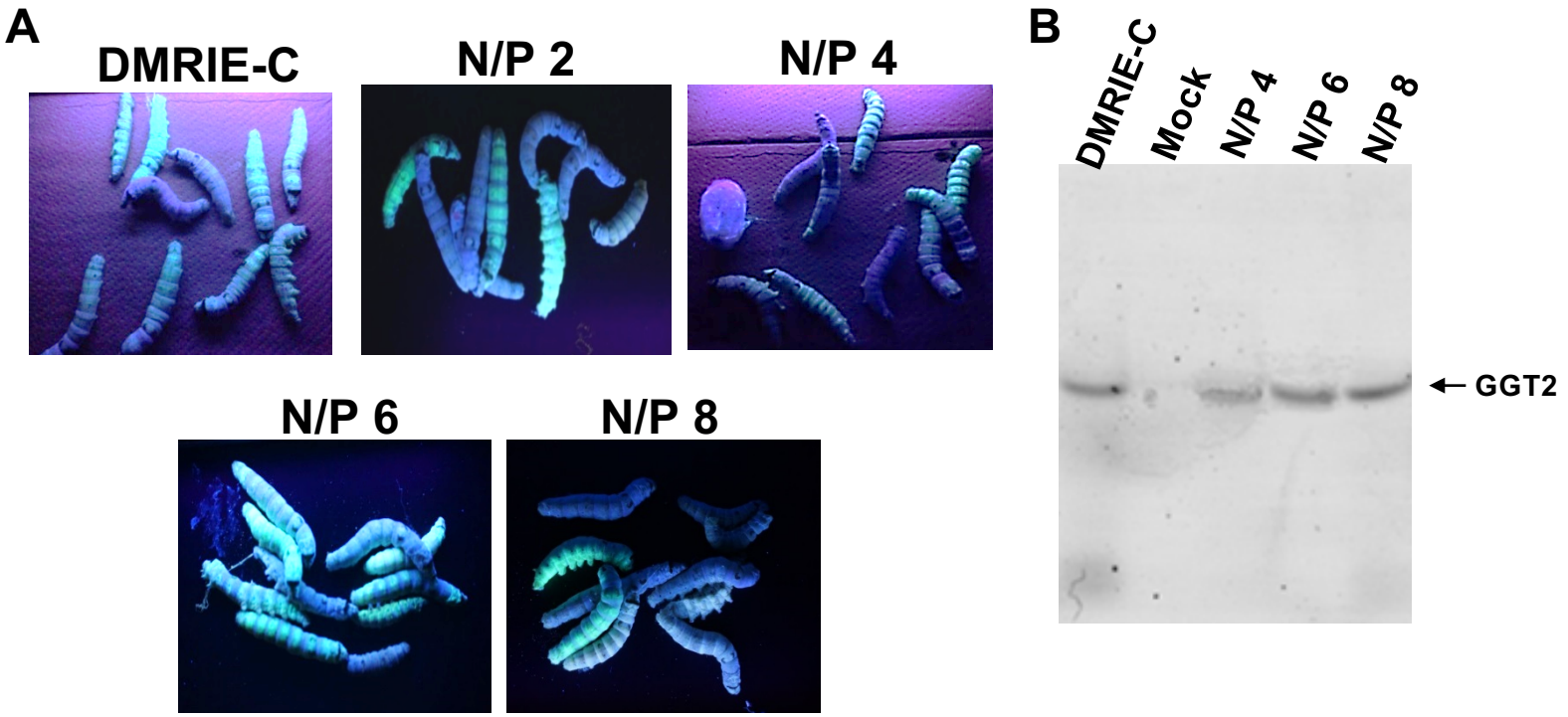

A
DMRIE-C
N/P 2
N/P 4
N/P 6
N/P 8
B
DMRIE-C
Mock
N/P 4
N/P 6
N/P 8
GGT2

Fig. 5 Kato et al.

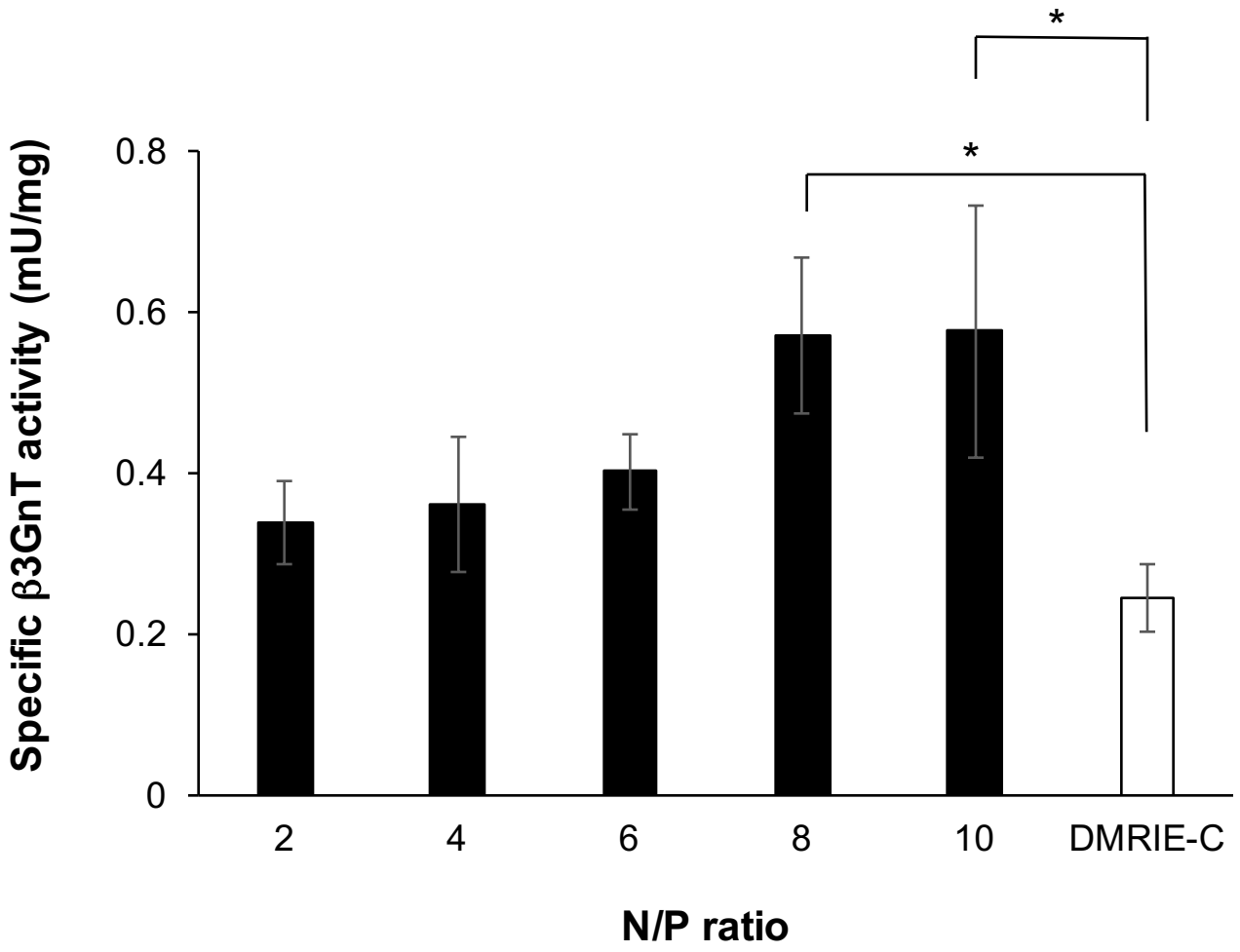


Fig. 6. Kato et al.