Baculoviral capsid display of His-tagged ZnO inorganic binding peptide

Lei Song · Yingying Liu · Jinchun Chen

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Abstract Virus-templated fabrication of compound structures can be made through incorporating the specifically inorganic-binding peptide into the viral scaffold, widely used is phage display system. Compared to prokaryotic phages, insect cell-based baculovirus has some strengths such as the adaptability to the proteins' posttranslational modification and non-replication in mammalian cells. As an attempt to explore the baculovirus-mediated bioconjugates, we show in this study that a genetically engineered baculovirus, with a hexahistidine (His₆) tagged ZnO binding peptide fused to the N-terminus of the viral capsid protein vp39 of AcNPV, was constructed. It maintains both the viral infectivity and the fusion protein's activity. The presence of the fusion protein on the baculovirus particle was demonstrated by western blot analysis of purified budded virus. Its display on the virus capsid was revealed by virus fractionation

analysis. The binding of nanosized ZnO powders to the virus capsid was visualized by transmission electron microscopy (TEM). This is the first report of the display of the inorganic-binding peptide on the capsid of eukaryotic baculovirus. Aimed at the nanomaterials' application in the biological field, this research could find useful in the biotracking of the baculovirus transduction process and the preparation of novel functional nanodevices.

Keywords Baculovirus · Genetic recombination · Inorganic-binding peptide · Capsid display · Bioconjugate

Introduction

The development of nanobiotechnology has made it very extensive the manufacturing of novel biocomposites, which provides attractive alternatives to the conventional synthetic methods in material science. Due to inherent recognition activity and self-assembly capabilities, biomolecules have been used as a desirable template for the synthesis with inorganics, particularly nanomaterials, to construct complex structures. Because of the nanoparticles' unique optical, optoelectronic and magnetic properties derived from their small size, these biocomposites could find valuable utilities in biological detection. The use of nanocrystals on DNA molecules (Mirkin et al. 1996; Mertig et al.

L. Song · J. Chen (✉)
College of Life Science and Technology, Beijing
University of Chemical Technology, 100029 Beijing,
China
e-mail: jinchunchen@hotmail.com

L. Song
e-mail: slof-songlei@163.com

Y. Liu
The Key Laboratory of Cell Proliferation and
Differentiation of Ministry of Education, Department of
Cell Biology and Genetics, College of Life Sciences,
Peking University, Beijing, China

2002), protein (Belcher et al. 1996) and viruses modified by metallic (Dujardin et al. 2003; Tseng et al. 2006) or semiconducting particles (Shenton et al. 1999) has been reported. Significant development has been made in the programmable assembly of hybrid nanoarchitecture by using genetically engineered virus (Mao et al. 2004; Lee et al. 2002; Huang et al. 2005). In these researches, more important is the immobilization of nanoparticles. For this purpose, substrate-specific peptide was incorporated into the virus scaffold to act as an ideal bioconnector.

In nature, there are many composite materials among which organisms and inorganics are well coordinated, such as mollusc shells (Falini et al. 1996) and animal teeth (Paine and Snead 1997). As we know, nucleation growth and assembly of in vivo minerals are regulated via protein scaffolds (Mann 1988; Sarikaya 1999). Informed by this biomimetic principle (Sarikaya et al. 2003) and with the aid of the combinatorial biology-based approaches originally used for screening peptides (Hoess 2001; Wittrup 2001), scientists have screened many desired inorganic binding polypeptides (Brown 1997; Naik et al. 2002; Sarikaya et al. 2004; Thai et al. 2004) using a cell or phage surface display system. These polypeptides with a length mainly ranging from seven to twenty amino acids are often referred to as genetically engineered polypeptide for inorganics (GEPI) owing to their high selectivity for inorganics. Recently, Zinc oxide as a unique semiconductive material with direct wide-bandgap (3.37 eV) and high excitation binding energy (60 meV), has been favorably used for developing optoelectronic devices. As an example of the study on bioinorganic composites, a GEPI derived from pJKS9 by Klemm's group (Kjærgaard et al. 2000) which specifically bound to ZnO was used here. In the former research, GEPI displaying outside the viruses (mainly prokaryotic phages), when genetically engineered into the viral coat proteins, can facilitate the nucleation and assembly of nanomaterials to generate hybrid conjugates with more functionalities (Huang et al. 2005). Similar to that, we report a novel genetically directed biosynthesis by incorporating the ZnO GEPI into the capsid protein of a eukaryotic baculovirus for the oriented assembly.

Autographa californica nuclear polyhedrosis virus (AcNPV) is a member of the family *Baculoviridae*, a group of large enveloped double-stranded DNA viruses and it was used here. Since the study of

Boublik's group in 1995 (Boublik et al. 1995), AcNPV has been developed as a useful vector of a eukaryotic viral display system. Mostly applied scheme was to enable the in frame-fusion of heterologous proteins to the amino-terminus of the baculovirus AcNPV major envelope glycoprotein gp64, which is introduced as a second copy of gene placed under the strong promoters of the viral late genes, polyhedron (*polh*) and *p10* dispensable for virus propagation (Grabherr et al. 1997, 2001; Mottershead et al. 1997). On infection, recombinant viruses with the foreign gene inserted within the gp64 copy at the *polh* or *p10* locus can synthesize large amounts of the fusion protein that gets displayed on the surface of the infected cell and the budded virions while maintaining their infectivity. Based on the above foundation of baculovirus expression system, another capsid display method has also been developed (Kukkonen et al. 2003), where foreign gene can be genetically designed to the N-terminus or C-terminus of AcNPV major capsid protein vp39 for recombinant expression under the control of *polh* promoter. It proved useful for biotracking of transgenes in gene therapy research. In this paper, we show the results of studies relevant to the construction of the recombinant baculovirus with His-tagged ZnO GEPI displayed on the viroosomal capsid by fusing it to the N-terminus of vp39, and its conjugation with nanoparticles under the help of GEPI binder.

Materials and methods

Baculovirus and host cells

Insect-derived *Spodoptera frugiperda* (Sf9) cells were maintained at 27 °C in TNM-FH Insect Medium (Sigma–Aldrich, St. Louis, MO), supplemented with 10% fetal bovine serum. The DH10Bac *Escherichia coli* competent cells were stored at −70 °C. Monolayers of Sf9 cells were grown on flasks and were infected by recombinant baculovirus at a multiplicity of infection (MOI) of 10. Then the products were analyzed 3 days post infection (p.i.). Virus stocks were stored at 4 °C.

Cloning of His-tagged ZnO GEPI gene

Based on the Klemm's pJKS9 sequence, four primers were designed to construct gene sequence expressing

ZnO GEPI. Primers used in the reaction were A1 (5'-GATCAAACACCCGCATGACCGCGCG-3'), A2 (5'-CCAGCATCGCTCCGCGAACCATAAAATCCA CCCAGCGCGCGTGAA-3'), B1 (5'-AGCTTTCACG CGCGCTGGGTGGATT-3) (stop codon shown in italics), B2 (5'-TATGGTTCGCGGAGCGATGCTG GCGCGCGGTCATGCGGGTGTTTT-3'), respectively. Viscous residues of *Bam*HI (C mutated to A) and *Hind*III designed at 5' terminus of A1 and B1 (underlined) were used for insertion. Four primers were mixed and *ddH*₂O-diluted to a 50 µL system containing 1 µL T4 PNK (T4 Polynucleotide Kinase), 5 µL T4 PNK Buffer. After phosphorylation and annealing, double-stranded fragments were synthesized and harvested by Recovery Kit (Invitrogen). Under the work of T4 ligase, as-prepared gene products were cloned into prokaryotic expression vector pET-28a(+) digested with *Bam*HI and *Hind*III, followed by transformation into competent cells DH5α. Finally, the plasmid bearing insert gene of ZnO GEPI, designated as PET-ZnO was screened and identified by sequencing analysis. His-tagged ZnO GEPI gene (*His-ZnO*) was obtained by polymerase chain reaction (PCR) amplification, using PET-ZnO as template and the following primers with *Bam*HI restriction site: forward primers, TTTGGATCCGG CAGCAGCCATCATCAT; reverse primers, TTTGG ATCCCGCGCGCTGGGTGGAT.

Construction of the transfer plasmid

Recombinant transfer plasmid was constructed using Bac-to-Bac Baculovirus Expression Systems (Invitrogen). The pFastBacTM1 vector was used as donor plasmid. At first, to generate AcNPV capsid display vector, the *vp39* gene (GenBank Accession No. M22978) was amplified by PCR from the purified bacmid DNA (Airenne et al. 2003) using the forward primer VF 5'-GGGAGATCTATGGGATCCGCGCT AGTGCCCGTGGG-3' (specific sequence for nt 469–485 of *vp39* gene in bold; *Bgl*/II and *Bam*HI sites underlined; start codon in italic) and reverse primer VR 5'-CCGTCTAGATCAGACGGCTATTCTC CACC-3' (specific sequence for nt 1489–1506 of *vp39* gene in bold; *Xba*I site underlined; stop codon in italics). The *Bgl*/II/*Xba*I-digested PCR product was cloned into *Bam*HI/*Xba*I-treated pFastBac 1 vector to get recombinant donor plasmid pFB-*vp39* with single *Bam*HI restriction site. Afterwards, the recombinant

transfer plasmid pFB-*vp39*-ZnO was constructed by the correct ligation of *Bam*HI-digested *His-ZnO* gene product with pFB-*vp39* also digested by *Bam*HI (as shown in Fig. 1). The resultant transfer plasmid was testified for accuracy by sequencing analysis.

Production and purification of recombinant budded baculovirus

Recombinant baculovirus was subsequently generated by transferring pFB-*vp39*-ZnO to bacmid within DH10Bac *E. coli* competent cells by site-specific

Fig. 1 Schematic presentation of the transfer plasmids used to construct BV-Zn recombinant viruses. Primers A1, A2, B1 and B2 were designed to synthesize a gene fragment encoding ZnO GEPI. It was inserted into pET 28a(+) plasmid and *His-ZnO* gene was obtained by PCR. The transfer plasmid was constructed by cloning of the *Bam*HI-digested PCR products into recombinant pFB-*vp39* vector. The insert gene was fused to the N-terminus of the *vp39* copy at the polh locus. The expression cassette of fusion gene was controlled by polh promoter

transposition, followed by transfection of recombinant bacmid DNA into Sf9 cells. Following the instruction of Bac-to-Bac system, recombinant virus named BV-Zn was harvested from the supernatant of the transfected cells and amplified by serial infections. An unrecombinant bacmid (Bac) was treated alike for use as the control. Recombinant baculovirus was purified by several rounds of centrifugation steps. Supernatant of infected Sf9 cells at 48 h.p.i. (hours post infection) was centrifuged at 5,000 g for 30 min at 4 °C (Sigma). The supernatant was centrifuged at 100,000 g for 1.5 h at 4 °C (Beckman, Ti70), then the virion pellet was resuspended in phosphate-buffered saline (PBS, pH 7.2) and stored at 4 °C as purified BV stocks.

Virus fractionation

Virus fractionation into nucleocapsids and membrane envelopes fractions was performed essentially as described previously (Braunagel and Summers 1994; Yang and Miller 1998). Briefly, purified BV virions were incubated in 1% Nonidet P-40 (Sigma), 10 mM Tris-HCl, pH 8.5, at room temperature for 30 min with gentle agitation. The samples were subsequently layered onto a 30% glycerol (w/v), 10 mM Tris-HCl, pH 8.5, and centrifuged at 150,000 g at 4 °C for 1 h. The envelope fraction was harvested from the top of the glycerol cushion, and the pellet containing capsids and part of the tegument was recovered as the nucleocapsid fraction and resuspended in PBS for further experiments. Intact BV-Zn virus and NP-40 treated virosome were made preparations for TEM observation.

SDS-PAGE and Western blot

Samples including purified recombinant virions BV-Zn, envelope and nucleocapsid fraction were solubilized in SDS-PAGE lysis buffer (0.5 M Tris-HCl, pH 6.8, 10% glycerol, 5% SDS, 5% β -mercaptoethanol, 0.25% bromophenol blue) and boiled at 100 °C for 5 min. Proteins were then separated by electrophoresis on 10% SDS-polyacrylamide gels, and transferred to PVDF membrane. The membrane was blocked with 5% milk in TBS for 30 min at 37 °C, and then incubated with mouse polyclonal anti-His antiserum (primary antibody, diluted 1:200) and mouse polyclonal antiserum to VP39 respectively

at 4 °C overnight. After being washed three times with TTBS (TBS containing 0.625% Tween 20), the membrane was incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Vector, diluted 1:200) as the secondary antibody for 1 h at 37 °C under continuous agitation. After additional three washes in TTBS, the blots were developed with BCIP/NBT substrates (Promega, Madison, WI) according to the manufacturer's instructions.

Prokaryotic expression and purification of the protein G-HZ

Using plasmid PET-ZnO DNA as template, and the following two primers: HZF forward sequence, 5'-GGGATCCGGCAGCAGCCATCATCAT-3' and HZR reverse sequence 5'-GCTCGAGTCAACGCGC GCTGGGTGGAT-3' (with *Bam*HI and *Xho*I sites underlined), *His-ZnO* gene was amplified by PCR. The *Bam*HI/*Xho*I-digested PCR products were ligated into appropriately cleaved vector pGEX-4T-3 and the resultant plasmid pG-HZ was then transformed into *E. coli* BL21 cells for expression under the induction of IPTG. The predicted fusion protein called G-HZ was purified through Ni-NTA system as described by ProBond Purification protocol.

Conjugation of membraneless virions with nanoparticles

Zinc oxide nanopowders with the size ranging mainly from 10 to 20 nm were presented by Dr. Peng Hu from Institute of Process Engineering, Chinese Academy of Sciences. The appropriately sized particles for microscopy were screened by several cycles of floating selection separation in distilled water. Finally, we get the uniform and smaller sized powder particles. For binding experiment, a few powders was put into an Eppendorf tube containing 1 mL distilled water and agitated homogeneously by sonication for 10 min. Then 20 μ L mixtures was added to the NP-40 treated membraneless recombinant BV-Zn virosome, which resuspend in 100 μ L purified G-HZ protein solution. Zinc oxide nanoparticles and envelope-free virions were mixed and allowed to bind to each other for 3 h at 4 °C laminar hood with gentle agitation. Afterwards, preparations of the mixture samples were subjected to TEM. Simultaneously,

unrecombinant Bac control virus was performed with the same procedures as above.

Electron microscopy analysis

The sample solutions were placed on collodion-coated 200 mesh copper grids with carbon reinforcement for 1 min and then negatively stained with 1% phosphotungstic acid (pH 7.0). TEM images were observed on a JEOL -2000EX operating at 80 kV after the samples were dried on copper grids with carbon reinforcement.

Results and discussion

Construction of transfer plasmid and recombinant virus

Schematic illustration of the transfer plasmid is shown in Fig. 1. The location of each primer and sizes of expected PCR products are indicated in Fig. 2. In short, the His₆-tagged ZnO GEPI gene *His-ZnO*, along with the baculovirus major capsid protein gene *vp39* was finally cloned into MCS of pFast-BacTM1 vector. The purposes of this design were based on two considerations: firstly, to express the fusion protein and display the target peptide on the baculovirus nucleocapsid; secondly, the existence of His₆ tag would facilitate the subsequent examination of identifying recombinant virus and make it possible to enable the purification of virions through a simple immobilized metal affinity chromatography (Hu et al. 2003). Correct construction of transfer plasmid was testified by Enzymatic digestion identification (Fig. 3a) and PCR detection (Fig. 3b), finally confirmed by sequencing analysis. So the foreign gene was fused to the N-terminus of the *vp39* copy at the *polh* locus. The expression cassette of fusion gene was controlled by *polh* promoter. The plasmid would efficiently produce the foreign peptide as N-terminal fusions with the baculovirus major capsid protein, *vp39*. With transferring the transfer plasmid into DH10Bac *E. coli* competent cells, recombinant Bacmid was obtained and transfected into the Sf9 cells to acquire recombinant baculovirus BV-Zn. The correct insertion of fusion genes into baculovirus DNA was confirmed by PCR analysis (Fig. 4).

Dissolution studies and localization of fusion proteins

To determine further that the fusion proteins were component of the viral capsids, we concentrated and purified the BV-Zn recombinant virus as above, then conducted dissolution studies using NP-40 detergent to remove the virion envelopes. After ultracentrifugation through a glycerol cushion of 30% (w/w), the pellet and supernatant fractions were analyzed on Western blots using mouse polyclonal anti-His₆ antiserum and mouse polyclonal antiserum to *vp39* capsid as primary antibody, respectively. The results were shown in Fig. 5. The presence of His₆ was detected in recombinant BV-Zn baculovirus nucleocapsid fraction (Fig. 5a, lane 1) but not in the envelope fraction (Fig. 5a, lane 2), which indicated the incorporation of the target peptide into the virus capsid protein *vp39* indeed. When three different virus samples were subjected to Western blot analysis using anti-*vp39* antibody, the results were shown in Fig. 5b as: lane 1 represents the recombinant virus which both expressed *vp39* protein and *vp39*-ZnO fusion protein, with two bands specific to anti-*vp39* antibody seen, which proved *vp39* fusion protein took part in the viral packaging in a certain incorporation rate and assembly of the viruses was not interfered; lane 2 shows no apparent blots in the envelope fraction; specific band relevant to the fusion protein was detected in lane 3, but indigenous *vp39* appeared not in the right position, the reason is not known yet. Therefore, based on the above Western blot analysis, it can be concluded that the His-tagged ZnO GEPI was exactly localized to the nucleocapsid of the recombinant virions such that it was not removed with NP-40 detergent.

Conjugation of particles and membraneless virosome

Recombinant BV-Zn baculovirus and nanoparticles were prepared, and fractionation was performed using nonionic NP-40 detergent treatment to harvest membraneless virosome (see materials and methods). Initially in the binding experiment, sonicated nanoparticles in the deionized water solution were put into the virus suspension in PBS buffer to allow incubation. However, no ideal results were received. The

Fig. 2 Schematic representation of construction of transfer plasmid and the location of each primer and sizes of expected PCR products. The region corresponding to the nucleotides (nt) 469–1506 of the vp39 gene (GenBank Accession No. M22978) was amplified from the bacmid DNA by PCR by using VF and VR primers. Codons inserted to generate *Bam*HI endonuclease

restriction site are *underlined*. The region corresponding to His tagged ZnO GEPI gene was amplified from the P-ZnO plasmid DNA where His tag codons are *boxed*. The locations of the primers, two primer sets used for PCR (VF and VR, HZF and HZR), and sizes of PCR products expected for the inserted regions (shown above *horizontal lines*) are indicated

Fig. 3 Restriction analysis and PCR identification of recombinant transfer plasmid. **a** Restriction enzyme analysis (*M*, *m*: DNA marker, *Lane 1*: recombinant plasmid digested by *Bam*HI and *Hind*III); **b** Identification of recombinant plasmid by PCR. *M*, DNA marker; *lane 1*, PCR product with forward primers of *His-ZnO* gene and reverse primers of *vp39* gene

main reason was probably attributed to the strong proclivity of nanoparticles to accumulate because of their high surface energy. To solve this problem, we attempted to express the peptide His-ZnO fused with Glutathione S-transferase (GST) in the pGEX-4T-3 prokaryotic expression system and purify the fusion protein called G-HZ using NTA-Ni purifying system

(Fig. 6). Better dispersivity of ZnO nanoparticles in the purified G-HZ protein suspension in elution buffer (ProBond Purification protocol) was attested. Upon that, we tried to resuspend NP40-treated recombinant virus capsids in the G-HZ protein suspension, followed by incubation with nanoparticles at 4 °C. Subsequently, sample preparations were

Fig. 4 PCR analysis of recombinant Bacmid, using forward primers of *His-ZnO* gene and reverse primers of M13. *M*, DNA marker; *lane 1*, PCR product

made for TEM analysis. As a control, unrecombinant Bac virions were also performed with the same treatment. Virus preparations including intact and unenveloped ones were observed by TEM, seen in Fig. 7a and b. The viral nucleocapsid showed a typical rod-shaped morphology. No apparent conjugation observed on the surface of unenveloped Bac control capsid (Fig. 7c), whereas certain particles could distribute around recombinant baculovirus nucleocapsid (Fig. 7d).

As a result, we incorporated the GEPI into the capsid of the baculovirus to construct a generic scaffold which can facilitate the construction of bioconjugates. Capsid-modified display system has proven a useful vector (Kukkonen et al. 2003) for baculovirus mediated gene therapy (Hu 2005, 2008). It may be helpful in the study of virus transduction mechanism in mammalian cells. In our work, we

Fig. 6 Purification of prokaryotically expressed G-HZ in recombinant pGEX-4T-3 plasmid. *M*, Protein marker; *lane 1*, SDS-PAGE results of the plasmid pG-HZ-induced bacterial proteins in the supernatant of lysate. *Lane 2*, Protein stainings of purified G-HZ fusion protein; *lane c*, control

successfully exposed His-tagged ZnO GEPI using this system. However, further investigations need to be made, including the influences of different nanomaterials of sizable and morphological distinction, the improvement of binding ambient and optimization method of getting the well-treated virus capsid and ideal composites. This design can be extended to other GEPIs and nanoparticles of interest. Combining with the usage of inorganic nanoparticles, particularly many quantum dots with unique optical properties, the generic GEPI-capsid displaying scheme would promise to be an alternative to transduction imaging detection used in gene therapy study, just like immunogold electron microscopy technology.

Fig. 5 Western blot detection of recombinant baculovirus with mouse anti-His and anti-vp39 polyclonal antibody, respectively. **a** Western blot analysis with mouse anti-His antibody. *M*, Protein marker; *Lane 1*, recombinant BV-Zn virus sample; *Lane 2*, envelopes fraction sample. **b** Western blot

detection with mouse anti-vp39 antibody. *M*, Protein marker; *lane 1*, intact recombinant intact virus sample with expressed vp39 and vp39-ZnO fusion protein; *lane 2*, envelope fraction sample of recombinant baculovirus; *lane 3*, nucleocapsids fraction sample

Fig. 7 Observation of recombinant BV-Zn baculovirus before and after fractionation, and visualization of conjugation of the control Bac virus and the BV-Zn (all envelope-free) with ZnO nanoparticles respectively by TEM. **a** Recombinant BV-Zn virus; **b** unenveloped BV-Zn baculovirus; **c** Binding situation of Bac nucleocapsids with nanoparticles; **d** Conjugation of BV-Zn nucleocapsids with ZnO nanoparticles. Bars, as represented

Conclusions

Summing up, in comparison with widely used phage display technology, a eukaryotic expression system is used for our study of inorganic–organic interactions. Capsid modification of AcNPV baculovirus by the introduction of His-tagged substrate-specific GEPI was successfully conducted. The vp39 protein was genetically modified and expressed using Bac-to-Bac system, resulting in the fusion of the substrate-specific peptide ZnO GEPI to the N terminus of the vp39 protein and display on the surface of baculovirus capsid. The recombinant baculovirus maintain both infectivity and the activity of the foreign peptide. Virus-based scientific research has attracted much more interest. Self-assembly and linkage of phenotype with genotype of virus display system make it a promising vector whether in medical or practical engineering applications. This insect cell-based eukaryotic baculovirus-GEPI generic framework, being safe and of economical interest, would be attractive new tool for immobilizing nanoparticles such as quantum dots for biological detection. It

allows further investigation into gene therapy study and novel functional devices.

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