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# Expression and self-assembly of *Heterocapsa circularisquama* RNA virus-like particles synthesized in *Pichia pastoris*

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*Heterocapsa circularisquama* RNA virus (HcRNAV) is the first single-stranded RNA virus to be characterized that infects dinoflagellates. The ability of HcRNAV coat protein (HcRNAV CP) to self-assemble into virus-like particles (VLPs) *in vitro* suggested that heterologous expression was possible, and that the VLPs might be ideal nanocontainers for the targeted delivery of genes and chemicals. In this paper, we report the expression of a codon-optimized *HcRNAV 109 CP* gene in *Pichia pastoris* and the production of self-assembled HcRNAV VLPs using large-scale fermentation. The *HcRNAV 109 CP* gene was synthesized according to the codon preference of *P. pastoris* and cloned into a pPICZA vector. The recombinant plasmid pPICZA-CPsyns was transformed into *P. pastoris* by electroporation. The resulting yeast colonies were screened by PCR and analyzed for protein expression by SDS polyacrylamide gel electrophoresis. After large-scale fermentation, the yield of HcRNAV CPsyns reached approximately 2.5 g $L^{-1}$ within 4 d. The HcRNAV VLPs were purified using PEG precipitation followed by cesium chloride density gradient ultracentrifugation, and were subsequently analyzed using UV spectrophotometry and transmission electron microscopy. Fluorescence dye-labeled myoglobin was loaded into the cages of the HcRNAV VLPs and the encapsulation was confirmed by fluorescence spectroscopy. The results point to the possible utilization in pharmacology or nanotechnology of HcRNAV VLPs produced by *P. pastoris* fermentation.

***Heterocapsa circularisquama* RNA virus (HcRNAV), virus-like particles (VLPs), expression, self-assembly, *Pichia pastoris***

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Harmful algal blooms (HABs), also known as red tides, have had a negative impact on aquatic ecosystems and have increasingly become a threat to human and marine health [1,2]. Rapid increases in an algal population can lead to water discoloration, shading of submerged vegetation, disruption of food-web dynamics and oxygen depletion in the water. HABs are known to have damaged the fishing industry, and to have affected shoreline quality and local economies. The potent neurotoxins can concentrate in filter-feeding shellfish and poison human consumers [3–5]. Even non-toxic algae can be harmful when they amass in sufficient numbers. HABs occur in many regions around the world, including Scandinavia, the North Pacific, the Caribbean and the South Pacific [6,7].

The toxic or harmful phytoplanktons that cause HABs are commonly dinoflagellates, such as *Alexandrium* and *Karenia* [8]. Most dinoflagellates have a unique structure that includes a nucleus known as the dinokaryon within which the chromosomes are attached to the nuclear membrane. Many efforts have been made to control harmful algal blooms with little success [9]. Today, the biological control of HABs is considered to be feasible [10]. Viruses that are abundant in marine systems replicate rapidly and tend to be host-specific, suggesting that single algal species

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could be targeted [11,12]. Parasites also have the potential to control algal bloom species, but their specific role in this regard is largely unknown [13].

*Heterocapsa circularisquama* RNA virus (HcRNAV) is the first single-stranded RNA virus to be characterized that infects dinoflagellates [14]. *H. circularisquama* Horiguchi, first observed in Uranouchi Bay, Japan, is a harmful bloom-causing dinoflagellate that specifically kills bivalves [15]. HcRNAV particles are polyhedral with a diameter of approximately 30 nm, and encapsulate a single positive-stranded 4.4 kb RNA genome. Two open reading frames (ORF-1 and ORF-2) were identified in the genome, ORF-2 coding for the viral coat protein [16]. HcRNAV targets and accumulates in the dinoflagellate nucleus. The virus clones have been divided into types CY and UA (HcRNAV109 and HcRNAV34, respectively), based on their host strain specificity [17].

The methylotropic yeast, *Pichia pastoris*, has been shown to be a suitable system for the heterologous expression of virus coat protein, which could then self assemble into virus-like particles (VLPs) *in vitro*. The successful examples include cowpea chlorotic mottle virus (CCMV), hepatitis B virus (HBV), and bacteriophage Qbeta [18–20]. Besides its ability to express foreign proteins at a high level, *P. pastoris* has been shown to have several advantages over other expression systems. The fermentation period was usually 4–5 d, compared to the plant hosts in which production took weeks [21]. The synthesized VLPs were soluble and able to self-assemble, while production in prokaryote hosts, such as *E. coli*, often results in insoluble inclusions [22].

In this paper, we report on the heterologous synthesis of HcRNAV 109 coat protein by *P. pastoris.* The successful large-scale fermentation and purification of the self-assembled HcRNAV VLPs suggest their potential application as nanocontainers. To our knowledge, this is the first paper to report the heterologous expression of HcRNAV.

## 1 Materials and methods

### 1.1 Synthesis of *P. pastoris* codon-optimized HcRNAV 109 CPsyns

The sequence of the *HcRNAV 109 CP* gene, GenBank accession [AB218609] [23], was redesigned to substitute amino acid codons that are seldom used in *P. pastoris* with those more frequently used [24]. Chemical synthesis of the new *CP* gene (*CPsyns*) was accomplished with GenScript, USA. Restriction sites for *Eco*R I (GAATTC) and *Not* I (GCGGCCGC) were added upstream and downstream, respectively, of the *CPsyns* gene. The plasmid pUC57-CPsyns was transformed into *E. coli* Top10 for amplification and preservation.

### 1.2 Construction of the recombinant vector pPICZA-CPsyns

The *P. pastoris* host strain GS115 (*his4*, histidine-requiring auxotroph) and the intracellular expression vector pPICZA were purchased from Invitrogen, USA. For the *in vivo* expression in *P. pastoris*, the 1.1-kb *CPsyns* gene was retrieved from the pUC57 vector as an *Eco*R I/*Not* I fragment and cloned into the corresponding *Eco*R I and *Not* I sites of the *Pichia* integrative vector, pPICZA. The resultant vector pPICZA-CPsyns was then transformed into *E. coli* Top10 for its amplification.

### 1.3 Transformation of *P. pastoris* and cultivation of HCRNAV 109 CPsyns

The transformation and expression of the *CPsyns* gene in *P. pastoris* was performed using established procedures [25]. The recombinant plasmid pPICZA-CPsyns was linearized with *Sac* I and subsequently used to transform *P. pastoris* GS115 by electroporation (Multiporator, Eppendorf, Germany). The transformed yeast cells were incubated in YPD agar containing Zeocin at 30°C for 2–3 d. Because the *CPsyns* gene was integrated into the 5′ alcohol oxidase promoter (AOX1) locus on the *Pichia* chromosome, all transformants would be $His^+$ $Mut^+$. The *CPsyns* gene in the transformed yeast cells was amplified with PCR using the CPsyns and AOX1 primers (data not shown).

Verified transformants were grown in a buffered glycerol-complex medium (BMGY) at 30°C for 16–20 h. The yeast cells were harvested using centrifugation and resuspended in buffered methanol-complex medium (BMMY) at an optical density of 1.0 at 600 nm (about $5\times10^7$ cells $mL^{-1}$). Incubation was continued for an additional 96 h at 30°C with 1% methanol induction every 24 h. To determine the optimal harvest time after induction, 1 mL of the expression medium was withdrawn every 12 h and the protein expression levels were determined using SDS polyacrylamide gel electrophoresis (SDS-PAGE). Non-transformed *P. pastoris* GS115 and cells transformed with the expression vector pPICZA/GS115 were included as controls for the same induction process.

### 1.4 Large scale fermentation of HcRNAV 109 CPsyns

A positive transformant GS115-24, reliably expressing the *HcRNAV CPsyns* gene, was selected to scale-up CPsyns production in a 5-liter fermenter (Fermentec XP 50, Korea) using established protocols (Pichia Fermentation Process Guidelines, Invitrogen, USA). A total of 10% of the initial fermentation volume of GS115-24 in BMGY was inoculated into BMGY in the fermenter. One mL of the fermentation medium was withdrawn every 12 h to monitor cell growth using absorbance at 600 nm. Coat protein expression levels were determined using SDS-PAGE. After the glycerol fed-batch phase and the methanol fed-batch phase, the culture was centrifuged to separate the cells from the supernatant. The harvested *P. pastoris* cells were placed on ice for further lysis, while protein concentration was analyzed using

the Bradford protein assay.

### 1.5 Purification of HcRNAV 109 VLPs

A modified virus capsid purification procedure based on PEG precipitation and density gradient centrifugation was employed to purify the soluble assembled HcRNAV VLPs [26]. The *Pichia* cells gathered by centrifugation at 4000×*g* for 30 min were resuspended in five times (w/w) lysis buffer (50 mmol $L^{-1}$ sodium phosphate, pH 7.4, 1 mmol $L^{-1}$ PMSF, 1 mmol $L^{-1}$ EDTA, and 5% glycerol) and disrupted by sonication (30 s on, 30 s off, 15 cycles, Vibra-cell VCX-750, Sonics, USA). Cell debris and insoluble material were removed by centrifugation at 10000×*g* for 30 min, and PEG 6000 was added to the supernatant to a final concentration of 10%. After overnight incubation at 4°C, the solution was centrifuged at 10000×*g* for 30 min and the resulting pellet was resuspended in phosphate buffer (10 mmol $L^{-1}$ $Na_2HPO_4$, 10 mmol $L^{-1}$ $KH_2PO_4$, pH 7.2). The resuspended PEG pellet was loaded onto a 10%–40% cesium chloride gradient and centrifuged at 35000×*g* for 2 h. The pellet was dissolved in the same phosphate buffer for further analysis.

### 1.6 Analysis of purified HcRNAV 109 VLPs

The purified HcRNAV VLPs were analyzed by UV spectrometry and transmission electron microscopy (TEM). Absorption at 260 nm and 280 nm was measured on a Scinco S-3100 UV spectrophotometer (Seoul, Korea), and the $A_{260}$∶$A_{280}$ ratio was calculated. TEM images of VLPs negatively-stained with 2%(wt:vol) uranyl acetate were obtained with a CM30 electron microscope (FEI/Philips) operated at 200 kV (Korea Basic Science Institute, Chunchon Center, Korea) [26].

### 1.7 Encapsulation of fluorescence dye-labeled myoglobin

Purified HcRNAV VLPs in PBS, obtained from Section 2.5, were dialyzed against three changes of disassembly buffer (0.05 mol $L^{-1}$ phosphate buffer, 0.15 mol $L^{-1}$ NaCl, 2 mmol $L^{-1}$ DTT, pH 8.0). Myoglobin was labeled with a fluorescent dye, Alexa Fluor 594, according to the Alexa Fluor® 594 Protein Labeling Kit (A10239, Invitrogen, USA). The disassembled HcRNAV VLPs and fluorescent myoglobin were mixed at the ratio of 5∶1 and the resulting mixture was incubated at room temperature for 1.5 h. The incubated solution was then dialyzed against three changes of reassembly buffer (0.05 mol $L^{-1}$ phosphate buffer, 0.5 mol $L^{-1}$ NaCl, pH 7.2) and concentrated by freeze-drying. Free myoglobin was removed using Vivaspin 6 ultrafiltration spin column (Sartorius, Germany). The VLPs containing encapsulated myoglobin were further analyzed and purified by size exclusion fast performance liquid chromatography (FPLC) on Biologic DuoFlow™ Chromatography System (Bio-Rad, USA) equipped with a Superose 6 PC 3.2/30 column (flow rate 40 μL $min^{-1}$, GE Healthcare, USA). The purified myoglobin-containing HcRNAV capsids were used to perform fluorescence spectroscopy (Confocal Laser Scanning Biological Microscope FV1000, Olympus, Japan) [27].

## 2 Results and discussion

### 2.1 Construction of expression vector pPICZA-CPsyns

The *P. pastoris* expression system was chosen due to its relatively simple fermentation protocol and high final cell density. To enhance the efficiency of gene expression, the HcRNAV CPsyns gene was optimized to the codon usage of *P. pastoris* (Figure 1). The synthesized 1080 bp sequence showed 95.6% nucleotide identity (1033/1080) and 100% deduced amino acid identity with the native *HcRNAV 109 CP* gene. The inclusion of seldom-used codons in *P. pastoris*, such as CGG (arginine) and GCG (alanine), can lead to low efficiency of gene translation via early termination. These codon alterations (CGG→AGA, GCG→GGT) were expected significantly to improve the expression level of foreign proteins. In total, 47 changes were introduced into the synthesized gene. The resulting HcRNAV CPsyns was then inserted into the pPICZA plasmid. The digestion of the recombinant vector by *Eco*R I and *Not* I showed two bands of 3.3 kb (pPICZA) and 1.1 kb (CPsyns), which indicated that the expression vector pPICZA-CPsyns was constructed successfully (Figure 2).

### 2.2 Expression of the HcRNAV 109 *CPsyns* gene in *P. pastoris*

After screening on Zeocin-YPD plates, over 10 *P. pastoris* transformants were induced with methanol and examined for HcRNAV CPsyns production. Each supernatant (5 μL) after cell lysis was subjected to SDS-PAGE (Figure 3). All of the transformants showed a band at approximately 40 kD, which was the theoretical molecular weight of the HcRNAV coat protein monomer. Transformant GS115-24 was selected for further fermentation.

The *P. pastoris* transformant GS115-24 grew exponentially for more than 40 h in the glycerol fed-batch phase in the 5-liter fermenter. Then, a methanol-fed induction was commenced over 72 h with the methanol concentration beginning at 3.6 mL $h^{-1}$ $L^{-1}$ and reaching 10.9 mL $h^{-1}$ $L^{-1}$. The maximum wet cell weight reached was 400 g $L^{-1}$ ($A_{600}$ of approximately 160). The concentration of crude HcRNAV coat protein approached 2.5 g $L^{-1}$ as determined by the Bradford protein assay, which was more than 12-fold the yield of HcRNAV CP in the shaking flasks. This is one of the highest productions of viral coat protein reported to date.

### 2.3 Purification of HcRNAV 109 VLPs

Recombinant GS115-24 cells were lysed by ultrasonic

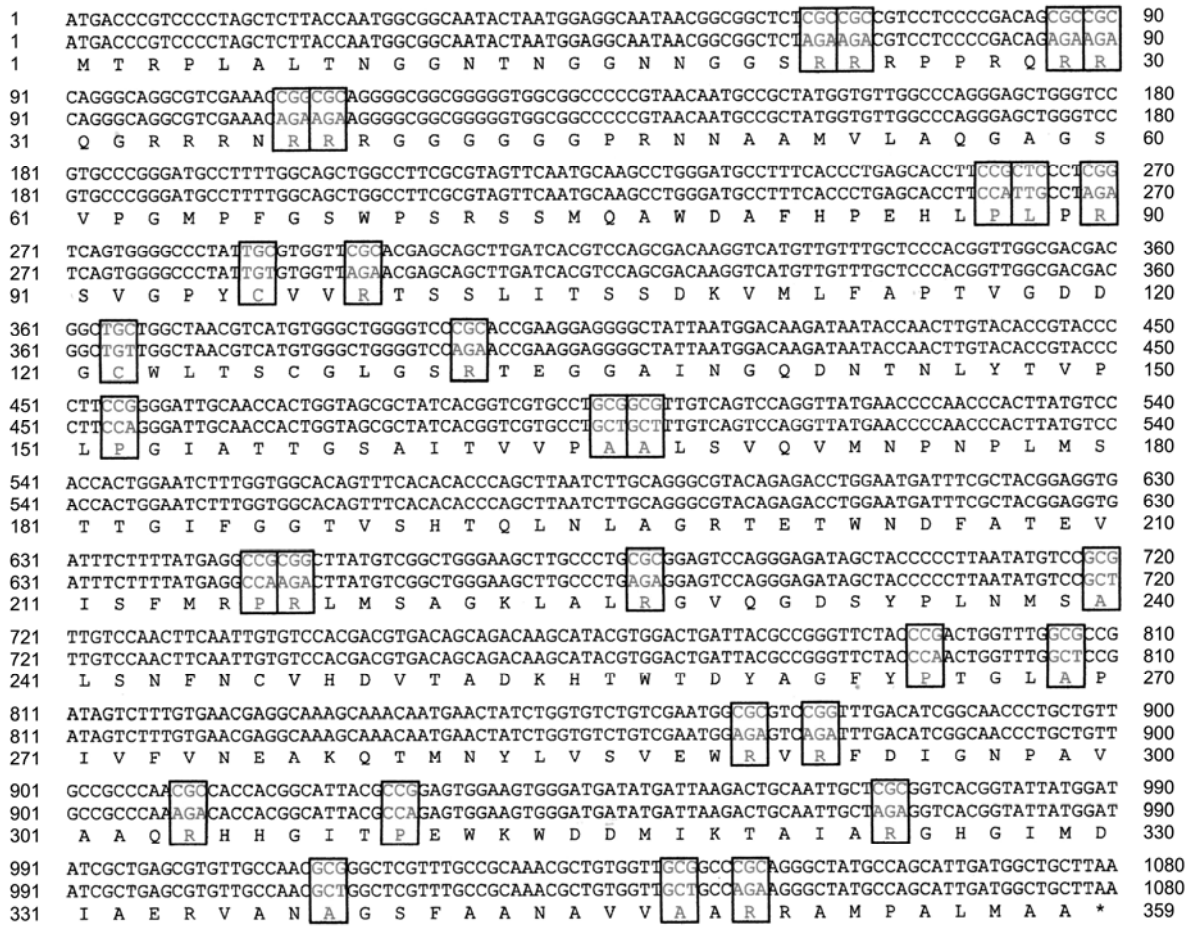

**Figure 1** Nucleotide sequences and deduced amino acid sequence of *HcRNAV 109 CP* gene (top line) and HcRNAV *CPsyns* gene (bottom line). The alterations in nucleotide sequence are boxed in gray.

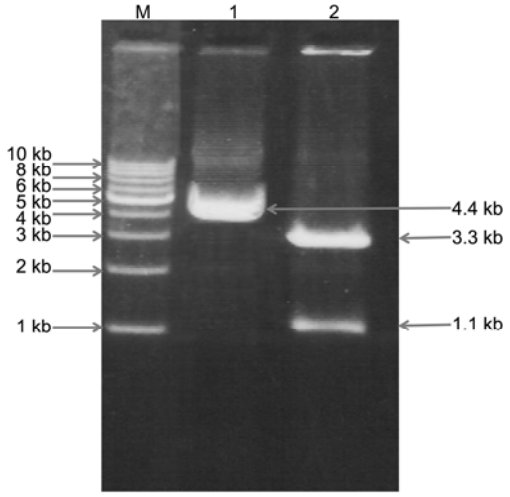


**Figure 2** Digestion of pPICZA-CPsyns with *Eco*R I and *Not* I. Lane 1, digestion of pPICZA-CPsyns with *Eco*R I; 2, double-digestion of pPICZA-CPsyns with *Eco*R I and *Not* I; M, Takara 1 kb DNA ladder.

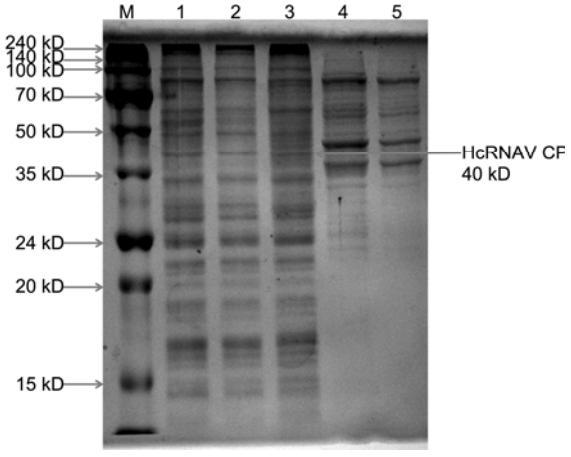


**Figure 3** SDS-PAGE of *P. pastoris* transformants. Lanes 1–3, soluble fractions of pPICZA-CPsyns transformants after sonication; 4, control of pPICZA/GS115; 5, control of GS115; M, DokDo-MARK$^{TM}$ broad-range.

disruption and the HcRNAV CP purified by PEG precipitation and density gradient centrifugation. PEG treatment of the supernatant preferentially precipitated HcRNAV CP with almost no precipitation of other proteins. Ultracentrifugation in CsCl further purified HcRNAV CP to a single band of 40 kD, which showed that the coat protein was able to assemble into high molecular weight structures (HcR-

NAV VLPs) (Figure 4).

### 2.4 Characterization of purified HcRNAV 109 VLPs

The VLPs were analyzed by UV spectrophotometry and showed an $A_{260}$:$A_{280}$ ratio of 1.51, which was similar to the ratio of 1.56 observed with other VLPs.

TEM examination of the VLPs showed spherical particles of ca. 30 nm diameter (Figure 5). This similarity to native HcRNAV particles indicated that the synthesized coat protein was successfully expressed and self-assembled into VLPs *in vitro* [14].

### 2.5 Encapsulation of fluorescence dye-labeled myoglobin

The co-elution of HcRNAV VLPs and guest myoglobin ($t$ = 36 min) was observed in the FPLC traces (Figure 6), showing the same elution time as the original capsids. These pointed to the successful reassembly of the viral cage after the encapsulation process.

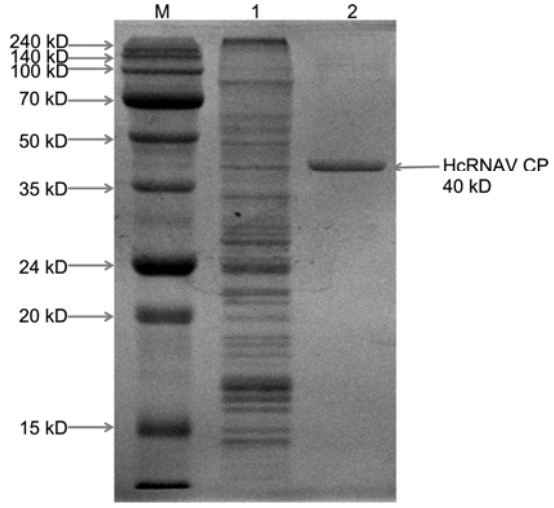


**Figure 4** Purification of HcRNAV coat protein. Lane 1, soluble fractions of GS115-24 after sonication; 2, HcRNAV CP purified by ultracentrifugation in CsCl; M, DokDo-MARK$^{TM}$ broad-range.

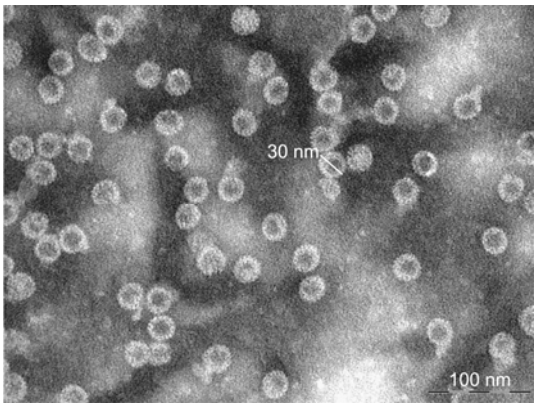


**Figure 5** TEM image of HcRNAV VLPs. The diameter of the capsids was about 30 nm. Size bar represents 100 nm.

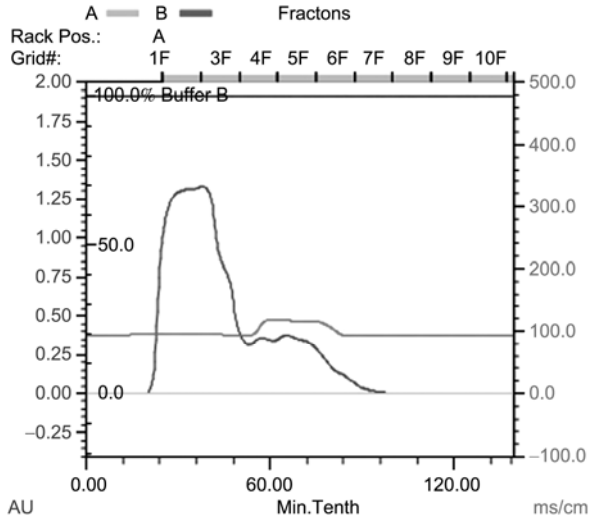


**Figure 6** Size-exclusion FPLC of myoglobin-containing HcRNAV VLPs at 280 nm. The peak at elution time of 36 min corresponded to the intact virus capsids.

The correct reassembly of HcRNAV capsids with fluorescent myoglobin was further proven by fluorescence spectroscopy. The capsids containing this guest protein displayed significant emission at $\lambda = 590$ nm (Figure 7), indicating that inclusion of myoglobin had occurred successfully. When HcRNAV VLPs were used as control, no emission under the same condition was observed.

## 3 Conclusion

HcRNAV 109 coat protein was expressed in *Pichia pastoris*

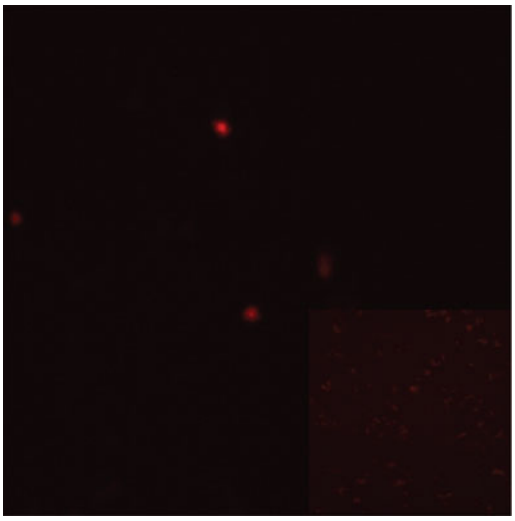

**Figure 7** Fluorescence spectroscopy of myoglobin-containing HcRNAV VLPs. A typical confocal fluorescence image (1.68 μm×1.68 μm) showed the formation of a fluorescent protein encapsulated inside a capsid. Inset: AFM image (to scale) at the same sample location, showing that only a small fraction of the capsids contained myoglobin molecules.

and successfully self-assembled into polyhedral virus-like particles. To our knowledge, this is the first report on the efficient expression and assembly in *P. pastoris* of the capsid of a virus infecting a harmful alga. Optimization of codons to those preferred by *P. pastoris* was necessary for the high expression of the foreign gene in the *P. pastoris* system [28]. The successful expression and purification of HcRNAV VLPs makes their abundant and rapid production now possible. This method also represents a promising tool for the preparation of large amounts of VLPs for use in gene therapy, vaccine development, and other applications such as the control of harmful algal blooms.

The encapsulation of myoglobin as a model protein inside HcRNAV VLPs revealed that these virus capsids derived from *P. pastoris* were suitable nanocontainers for drug delivery and imaging reagent carriers. The specific targeting by HcRNAV of *H. circularisquama* would be extremely useful in controlling harmful algal blooms. The encapsulation of algicidal compounds inside the capsids to improve the efficiency is another useful approach for future investigation. HcRNAV could become a model virus capsid that might be used as a nanoplatform in materials science and medicine. Further analysis to compare the properties of the recombinant HcRNAV capsids with those of the native types is ongoing. The modification of HcRNAV CP by altering certain functional amino acids could also be useful in revealing the detailed structure, currently unknown, of the HcRNAV capsid.

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