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G．知的財産権の出願・登録状況（予定も含む）

なし

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Critical Review

Engineered Bio-nanocapsules, the Selective Vector for Drug Delivery System

Dongwei Yu[1], Takayuki Fukuda[1,2], Tuoya[1], Shun'ichi Kuroda[3,6], Katsuyuki Tanizawa[3,6], Akihiko Kondo[4,6], Masakazu Ueda[5,6], Tadanori Yamada[6], Hiroko Tada[1] and Masaharu Seno[1,6,7]

[1]*Graduate School of Natural Science and Technology, Okayama University, 3.1.1 Tsushima-Naka, Okayama 700-8530, Japan*
[2]*MRP Division, Katayama Chemical Industries Co. Ltd., 2.5.10 Doshomachi, Chuo-ku, Osaka 541-0045, Japan*
[3]*Institute of Scientific and Industrial Research, Osaka University, 8.1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan*
[4]*Faculty of Engineering, Kobe University, 1.1 Rokkodai, Naka, Kobe, Hyogo 657-8501, Japan*
[5]*Keio University, School of Medicine, 35 Shinanomachi, Shinjuku-ku. Tokyo 160-8582, Japan*
[6]*Research Center, Beacle, Inc., Okayama Research Park Incubation Center, 5303 Haga, Okayama 701-1221, Japan*
[7]*Research Center for Biomedical Engineering, Okayama University, 3.1.1 Tsushima-Naka, Okayama 700-8530, Japan*

Summary

The bio-nanocapsule (BNC) is our concept of artificial hollow nanoparticles that have been designed and produced through biotechnological procedures. We proposed an empty virus-like particle, which consists of a recombinant L envelope protein of hepatitis B virus (HBV) and a lipid derived from the host cell, as an engineered BNC. Although this BNC was first developed as an immunogen of hepatitis B vaccine, the pre-S1 region in N-terminus of L envelope protein confers hepatocyte specific infectivity of HBV on the BNC. This recombinant BNC is now being developed as a novel platform of drug delivery system (DDS) vector for selective delivery.

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Keywords Vector; specific infection; delivery; topology.

INTRODUCTION

The drug delivery system (DDS) is one of the areas of intense focus recently in pharmacology. However, the development of DDS technology has concentrated on the controlled release of pharmaceutical drugs encapsulated in a nano-sphere composed of such substances as micelles and liposomes. The targeting potential of the pinpoint delivery in DDS has appeared to be ignored since antibody-based 'missile therapy' has been extensively studied by immunologists. Although live viruses are sometimes toxic or oncogenic to the infected cells, they have been found to be extremely useful for the transfer of genes to the cells. Consequently, the development of artificial viruses as vectors for gene transduction or gene therapy has caused vigorous research worldwide as a part of nanobiotechnology. This new area of science deals with molecules as nanostructures and molecules in nanostructures such as nanoparticles not only for the conventional analyses of molecules but for the extensive development of new tools in biotechnology and medical engineering (*1*). In presenting an overview of the viruses, it may be noted that many of them are known to have host ranges, but to rarely exhibit cell type or tissue type selectivity. Only a few of them exhibit some narrow range of cell type selectivity like HBV or HIV, for example. The HBV is among the best known viral pathogens that affect humans. It has been studied for more than 60 years in the hope of learning how to prevent its infection. HBV is a small enveloped DNA virus of the hepadnaviridae family (*2, 3*). The surface proteins of the virion envelope are found to consist of three types of glycoproteins, which are called small- (S-), middle- (M-) and large- (L-) protein (*4*). These three different proteins, which are cotranslationally integrated into the rough endoplasmic reticulum as transmembrane proteins, are encoded in a single open reading frame of HBV genome with three independent, but in-frame, AUG translational start codons (*5, 6*). The HBV infected cells secrete, not only the complete 42-nm infectious virions, but also a large excess of 22-nm noninfectious empty envelope particles. The latter are composed of S-protein (*7–9*). Valenzuela et al. successfully prepared HBV surface antigens (HBsAg) as recombinant S-protein, which organized itself as a particle having a diameter of 22 nm (*10*). When produced in

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Address correspondence to: Masaharu Seno, Graduate School of Natural Science and Technology, Okayama University, 3.1.1 Tsushima-Naka, Okayama 700-8530, Japan. Tel/Fax: +81 86 251 8216.
E-mail: mseno@cc.okayama-u.ac.jp

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yeast, recombinant M- and L-proteins were also found to associate as hollow virus-like particles, although they had been only developed as immunogens for hepatitis B vaccines (*11, 12*). The virus-like particle composed of the recombinant L-proteins had a diameter of approximately 200 nm (*12, 13*). Due to the specific affinity to human hepatocytes localized in the amino-terminus of L-protein, only the nanometer size hollow particle, composed of L-proteins, showed extremely selective targeting potential as a novel type of DDS vector to be directed to the human liver (*14*). Because of its favorable potential, we have proposed a bio-nanocapsule (BNC) concept as an efficient nanomachine to achieve tissue-/cell-type specific delivery of genes, drugs and proteins.

PROPERTIES OF ENGINEERED BIO-NANOCAPSULES

Our BNC is described as a hollow protein nano-scale particle. Some viral core particles, which consist of sole self-organizing proteins, such as HBV core antigen (cAg) and others that also consist of self-organizing proteins with lipids as essential components, like the envelope of hemaglutinating virus of Japan (HVJ), are conceivable. Since the BNC should function as a capsule that contains some encapsulated foreign substances in its interior, the lipid component appears to be essential to confer flexibility in order to maintain a suitable shape as a DDS vector even after the physical implementation of encapsulation such as electroporation. In this context, HBV cAg does not appear to be suitable as a capsule due to the tight association of each component of cAg. The HBV envelope proteins form a BNC, since almost 80% of the components are L-, M- or S-proteins. This BNC of HBV can be formed by a single species of viral surface proteins. Therefore, newly engineered BNC can be designed and produced by modifying the protein in a variety of ways by a simple procedure. The L-protein is composed of three regions, the 108 or 119 amino acids (subtype ayw or adw, respectively) pre-S1 region (*8, 15*) involved in the direct recognition of human hepatocytes, the 55 amino acids pre-S2 region associated with the polymerized albumin-mediated interaction (*16*), and the major 226 amino acids S protein region.

When L-protein was expressed in yeast cells, it failed to assemble into a subviral particle, unlike S protein, although the protein is efficiently expressed (*17, 18*). The synthetic gene for chicken-lysozyme signal (C-SIG) peptide was fused at the 5′-terminal of L gene. The C-SIG peptide was processed correctly by the yeast secretory apparatus and led the pre-S1 region in traversing the ER membrane. Then, the L-proteins assembled themselves into a virus-like particle as a L-BNC. A small region of $Ser^{44}-Thr^{48}$ containing a site sensitive to the trypsin-like protease ($Arg^{48}-Thr^{49}$) has been deleted from pre-S2. The addition of C-SIG peptide and deletion of $Ser^{44}-Thr^{48}$ did not affect the stability of L-protein and polymerized human serum albumin binding activity (*19*).

This engineered L-protein, produced as a L-BNC in recombinant yeast cells, has been purified and characterized. The average molecular mass of a L-BNC was confirmed by equilibrium sedimentation to be 6.4×10^6, which indicates that about 110 molecules of L-protein associate to form an L-BNC. Atomic force microscopy in a moist atmosphere showed the L-BNCs to be spherical particles with diameters ranging from 50–500 nm and an average of 200 nm. The L-BNCs were stable at a high temperature of around 80°C and, for a week, at 4°C, but were found to become unstable as a result of freezing and thawing or treatment with dithiothreitol (*19*).

ENGINEERED BIO-NANOCAPSULE HAS THE SAME SPECIFIC INFECTIVITY AS HBV

HBV, a member of hepadnaviridae, known as a family of hepatotropic, has a very narrow host-range and is limited to infecting naturally humans and some higher primates, such as the chimpanzee (*11, 20, 21*). It has been demonstrated that the L-protein is important in forming an infectious virus particle and essential for receptor interaction (*22–24*). In particular, the 108 or 119 amino acids of pre-S1 domain of glycosylated L-protein have long been considered to be responsible for receptor binding and the host range (*22, 25, 26*), while the 55 amino acids of pre-S2 is dispensable for viral infectivity (*27*). The involvement of L-protein in the infection stage has been confirmed. Furthermore, the segment from 21 to 47 amino acid of pre-S1 was found to be indispensable for HBV infection (*28–31*). However, the latest research demonstrated that more amino acid residues, extending from 3 to 77, are involved in this process (*22*). The pre-S1 region of the L-protein, displayed on the surface as the specific ligand for receptor of human hepatocytes, certainly confers on the L-BNC the high infectivity to hepatocytes limited to humans and primates. Very recently, we have shown that the L-BNC efficiently delivers the gene for green fluorescence protein (GFP) and GFP protein to human liver cells in a cell type specific manner *in vitro* and *in vivo* (*14, 32*). Thus, our engineered L-BNC was demonstrated to be an efficient delivery mimicking HBV.

ENGINEERED BIO-NANOCAPSULE CAN DELIVER SUBSTANCES TO TARGET TISSUE OR CELL

HBV is the only DNA virus that targets the liver where it efficiently infects hepatocytes. This ability permits the development of an HBV-based vector for liver-directed gene transfer with foreign DNA inserted into the virus genome (*33, 34*). However, this viral vector is not suitable for the delivery of substances other than DNA. Since the L-BNC is an empty particle that recognizes human hepatocytes just like HBV, electroporation was used to encapsulate the DNA and small chemical compounds that were inside the L-BNC (*14*). The GFP gene or fluorescent chromophore calcein was successfully encapsulated by electroporation and transferred

to human hepatocytes in culture, or in a xenograft model with the specific infection of the L-BNC. This specificity was shown by the green fluorescence observed only in human hepatocellular carcinomas. Any other cells of human carcinomas or of host tissues did not show positive fluorescence. When the gene for human clotting factor IX was transferred into the xenograft model by the L-BNC, factor IX was produced at a significant level in the sera relevant to the treatment of moderate hemophilia B.

Although electroporation is proposed as one of the procedures suitable for enclosing substances in the particle (*14*), optimal conditions for electroporation appear to be lacking. The empty particles that failed in encapsulation must be removed just prior to infection in order to attain the highest delivery efficiency. If not, they will bind to receptors on the surface of hepatocytes competing with the particles carrying the substances, resulting in low transfer efficiency. A more efficient procedure might be required in place of electroporation.

In the case of delivery of proteins by the BNC, it appears to be difficult sometimes to introduce the proteins into hollow particles with canonical electroporation due to the high-voltage pulse and disordered redox conditions that denature the proteins. The fusion strategy was proposed as a suitable procedure to prepare the BNC that delivers proteins to human hepatocytes. This strategy will ensure the generation of protein for transfer accompanied by the formation of recombinant L-BNCs. The gene of EGFP, an analogue mutant of GFP, fused to the 3' end of L gene, was expressed in mammalian COS-7 cells and the BNCs of L-EGFP fusion were produced and prepared. When infected, these L fusion particles showed green fluorescence only on the human hepatocytes (*32*), indicating that the L fusion particles have equivalent specific transfection efficiency due to the hepatocyte recognition site present in the pre-S1 region. No encapsulation procedure, such as electroporation, is required if the fusion strategy is available for the BNC. All of the particles are destined to convey the protein of interest with a transfection efficiency of nearly 100% to human liver cells.

Thus, it has been demonstrated that the BNC is an ideal vector for the cell- or tissue-type specific delivery of genes, drugs and proteins *in vitro* and *in vivo*. Together with the advantage that it is mass produced in yeast cells and free from viral genomes, the BNC should be an extremely useful novel vector system, which could be introduced to clinical trials in the near future (Fig. 1).

UNIQUE C-TERMINAL TOPOLOGY OF L-PROTEIN IN ENGINEERED BIO-NANOCAPSULE

The topological information of the HBsAg envelope protein came primarily from the research of S-protein (equivalent to the S domain of L-protein). L-protein was found to have a dual N-terminal topology. The N-terminal pre-S domain of L-protein functions as an adapter for a virus receptor when it is located on the surface of a secreted virion particle. While the pre-S domain is located inside a viral envelope, it is believed to function as a matrix protein that mediates contact between viral envelope and nucleocapsid during virion assembly (*35–37*). Two hydrophobic regions in the S domain enable the L-protein to traverse the membrane at least twice. The hydrophilic region flanked by the two hydrophobic regions is considered to be located on the interior side of the BNC. The major HBsAg epitopes in the S domain following the second hydrophobic region are located on the

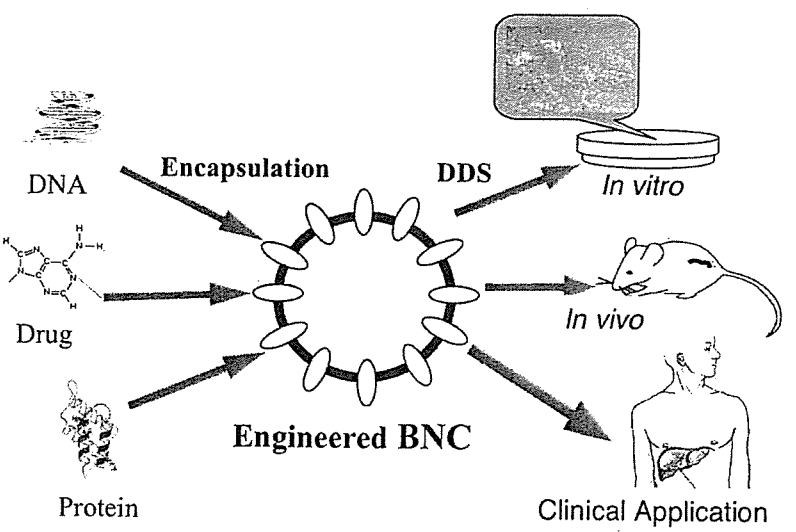

Figure 1. The concept of the application of engineered BNC for DDS. The BNC has the potential to incorporate DNA, drugs and proteins in its interior and to transfer them in a cell-/tissue-type specific manner *in vitro* or *in vivo*.

surface of the BNC. However, there has been no detailed critical description of the hydrophobic C-terminus region of the S domain to date. Eble et al. provided experimental evidence that the C-terminus protrudes from the particle (*38*). Localization of HBV epitope by monoclonal antibodies against HBV surface antigen suggested that epitope from 178 to 186 amino acid of S domain is exposed on the surface of the virion particle (*39*).

During the design of the fusion of the BNC and EGFP, FLAG-tag and EGFP were fused to the C-terminus of L protein with some length truncated. The fusion particles of L-FLAG-EGFP could be immunoprecipitated with the anti-FLAG antibody and anti-GFP antibody (*32*). These results also indicate that the C-termini of L located at the external side of the particle is suitable for a single spanning in the membrane even after the truncation of the C-terminus. However, the protease protection assay revealed the presence of another independent C-terminal topology of L-protein. The results showed that L-EGFP fusion protein takes a dual C-terminal topology, displaying EGFP moiety outside of, or enclosing it inside, a BNC. However, the experiments showed that only single orientation characterizes a particle (Fig. 2).

The dual C-terminal topologies of the fusion BNCs could be applied to various proteins for the C-terminal moiety of the fusion, depending on the character of the proteins. They might include cytoplasmic proteins, as well as cytokines or ligands to cell surface receptors. This fusion design is considered to be the most efficient way to prepare a BNC that delivers proteins to specific cells or tissues.

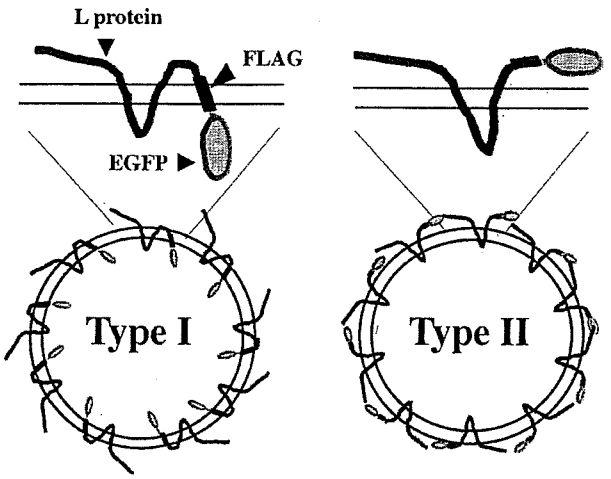

Figure 2. The C-terminal of the EGFP moiety in L-protein fusion has dual topologies. The flexible C-terminus topologies result in the formation of two types of the L-BNC-EGFP. Each of these topology types occupies a particle independently. Type I, EGFP moiety is encapsulated inside; type II, EGFP moiety is displayed outside.

THE TARGET OF ENGINEERED BIO-NANOCAPSULE CAN BE ALTERED

The pre-S1 domain in L-protein contains a specific host cell human hepatocyte recognition site present at the sequence of 3 to 77 amino acid of HBV subtype ayw (*22*, *23*). When the nucleotide sequence in L-protein, which corresponds to this site, was substituted with a synthetic gene encoding 53 amino acids of mature human epidermal growth factor (EGF), the EGF-BNC was expressed and found to display the EGF moiety on the surface of particles. The EGF-BNCs incorporating calcein were mixed with A431 cells, which overexpressed EGF receptors, or hepatic NuE cells. A high level of fluorescence was observed only in A431 cells, whereas no fluorescence was observed in NuE cells, indicating that the EGF moiety functions to target cells in a specific manner (*14*). This result demonstrates that the pre-S1 region in L-protein plays a critical role in cell recognition and that foreign biorecognition molecules can be substituted to alter the specificity of BNCs. Now, we are developing a convenient BNC that has the specific binding site for hepatocytes substituted with a peptide derived from protein A with a specific affinity to the Fc region of IgG molecules (Muraoka et al. manuscript in preparation). Due to the specific affinity to the antibody, the specific tropism of the engineered BNC could be easily altered, depending on the antibody displayed on the surface of the BNC. Recently, we successfully designed a DNA microarray for the screening of cell surface markers (*40*). In the screening of the cell surface marker of SV40 transformed mouse fibroblasts, we found that the expression of CD62L and IL-6 receptor genes were upregulated, in comparison to normal fibroblasts. The antibodies for these markers might be available to target these tumor cells, for example. The search for the cell type-specific surface markers would become a very important component of any efforts to improve the availability of the BNC.

FUTURE PERSPECTIVES

There are 14 cysteine residues in the L-protein, and they are localized only in the S domain. Since Cys48, Cys65 and Cys69 were found to be essential for the secretion of particles (*41*), the other cysteine residues were considered to be the cause of the oligomeric form of L-proteins induced by false intra- or inter-molecular disulfide bonds. We are now attempting to remove the unessential cysteine residues and 8 have been successfully removed (Fukuda et al. submitting to J. B. Chem). The multiple mutations in L-protein enhanced the stability of the BNC and raised the efficiency of encapsulation ameliorating the specific infectivity to target cells.

Because of the worldwide Hepatitis B vaccination program, the application of the BNC will be limited in clinical application. In L-protein, the segment extending from 99 to 169 amino acid of S domain contains a highly conformational

epitope cluster (*42*). The 'a' determinant region extending from 121–149 amino acid especially induces typical protective neutralizing anti-HBs antibodies. It will be important to arrange the sequence in the S domain for engineered BNC, which has less immunogenicity, as a safe BNC. With these sophisticated improvements, the BNC should be extremely useful vectors for academic research and clinical applications.

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Development of Novel Yeast Cell Surface Display System for Homo-oligomeric Protein by Coexpression of Native and Anchored Subunits

Hirotaka Furukawa,[†,‡] Takanori Tanino,[‡] Hideki Fukuda,[‡] and Akihiko Kondo*[,§]

Yokohama Research Center, Chisso Corporation, 5-1 Ookawa, Kanazawa-ku, Yokohama 236-8605, Japan, Division of Molecular Science and Material Engineering, Graduate School of Science and Technology, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe 657-8501, Japan, and Department of Chemical Science and Engineering, Faculty of Engineering, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe 657-8501, Japan

Streptavidin derived from *Streptomyces avidinii* was displayed on the cell surface of the yeast *Saccharomyces cerevisiae* by cell-surface engineering using two types of plasmid for the expression of a native subunit and an anchored subunit fused with the C-terminus of 318 amino acids of Flo1p containing a glycosylphosphatidylinositol anchor attachment signal. The displayed streptavidin had the binding ability for biotinylated compounds. This was confirmed by fluorescence microscopy after the adsorption of yeast cells displaying streptavidin and biotinylated fluorescein isothiocyanate. On the other hand, streptavidin produced by cells harboring only the plasmid for the expression of the anchored subunit showed a very low binding activity for biotinylated compounds. Cells displaying streptavidin may constitute novel whole-cell affinity adsorbents widely used for immunoassay and biosensing. This coexpression method will ensure that proteins, such as homo- and hetero-oligomeric proteins, are displayed on the cell surface in an active form.

Introduction

There have been numerous studies of expression systems for the display of heterologous proteins on the cell surface of microorganisms (*1*−*5*). It has much potential for various applications, such as whole-cell biocatalysis and combinatorial library construction. In particular, the expression of heterogeneous proteins on the cell surface of the yeast *Saccharomyces cerevisiae* has been studied extensively (*2*, *6*−*11*). To display proteins on the cell surface of *S. cerevisiae*, native cell wall proteins, such as α-agglutinin and Flo1, with a serine/threonine-rich hydrophobic tail for glycosylation and a glycosylphosphatidylinositol (GPI) anchor attachment signal at the C-terminus have been used (*6*, *8*, *9*, *11*).

However, the use of anchor proteins has restricted the types of expression protein. Namely, monomeric proteins have been expressed mainly because the anchor proteins control the direction of display. Figure 1A shows two possibilities when a homo-oligomeric protein is expressed on the cell surface by the conventional method. The possibility of the expressed protein to have the proper activity in the case of (i) is low because of the anchor protein. On the other hand, the expressed protein has no activity in the case of (ii). To develop a new method, we have established the coexpression system shown in Figure 1B. In this system, the displayed subunit would become an active form by assembling with the coexpressed native subunit with the signal peptide. To prove the feasibility of our concept, attention was paid to a tetrameric protein, streptavidin (M_w = 60 000) produced by *Streptomyces avidinii*. Streptavidin binds to a soluble vitamin, D-biotin, with a markedly high affinity

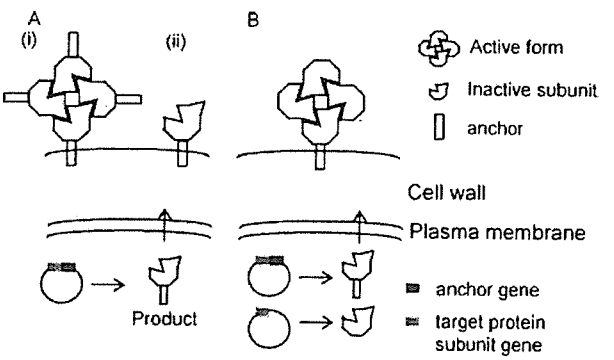

Figure 1. Schematic illustration of possible states of a homotetrameric protein expressed on the cell surface by the conventional method (A) and the novel method (B). Conventional method allows subunits anchored on the cell wall to assemble into tetrameric form incidentally (A-i) or stay in monomer form (A-ii). Novel method allows subunit anchored on the cell wall to assemble into a tetrameric form with secreted subunits.

(*12*, *13*). The display of streptavidin on the yeast cell surface is an important breakthrough. This is the first report of a display of a homo-oligomeric protein on a cell surface which is effective for immobilizing biotinylated functional compounds in the industrial field. As the anchor protein for cell surface display, the C-terminus of 318 amino acids of the Flo1 protein (Flo318) was used. Here, we report for the first time the development of a novel technique for displaying a homo-oligomeric protein on the cell surface.

Materials and Methods

Strains and Media. *Escherichia coli* strain Novablue {*endA1*, *hsdR17* (rk_{12}/mk_{12}^+), *supE44*, *thi-1*, *recA*, *gyrA96*, *relA1*, *lac* [F′ *proA*$^+$*B*$^+$ *lacIqZ*Δ*M15*::Tn*10*(Tc^R)]} (Novagen, Inc., Madison, WI) was used as the host for recombinant DNA manipula-

* To whom correspondence should be addressed. Tel: +81-78-803-6196. Fax: +81-78-803-6206. E-mail: akondo@kobe-u.ac.jp.
† Chisso Corporation.
‡ Graduate School of Science and Technology, Kobe University.
§ Faculty of Engineering, Kobe University.

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tion. *S. cerevisiae* strain MT8-1 (*MATa, ade2, his3, leu2, trp1, ura3*) (*14*) was used for breeding the yeast strain. *E. coli* was grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 0.5% sodium chloride; w/v) with 100 μg/mL ampicillin. Yeast was precultivated in a selective medium (SDC: 0.67% yeast nitrogen base supplemented with appropriate amino acids and nucleotides, 2% glucose, and 2% casamino acid) at 30 °C for 30 h. Then yeast cells were inoculated in 100 mL of SDC medium (initial OD_{600} = 0.03) and aerobically cultivated at 30 °C.

Construction of Plasmids. Plasmids for the cell surface display and the extracellular expression of streptavidin were constructed as follows. The gene encoding the core region of streptavidin was amplified from the genome of *S. avidinii* IFO 13429 by polymerase chain reaction (PCR) using the following primers: 5′-AGACGTCGACCATGGAGCCGAGGCCGGCATCACC-3′ and 5′-AATCCCGGGCTCGAGAAGATGGAGGCGGCGGACGGC-3′ for the cell surface display. The amplified fragment was digested at the *Sal*I and *Sma*I sites and inserted into the *Sal*I and *Sma*I sites of pUI3αEGFPFlo318 (*15*). The resultant plasmid was named pWI3αSAFlo318. The same gene was amplified from the same genome by PCR using the following primers: 5′-GAGACGTCGACCATGGCGCCGAGGCCGGCATCACCGGCACCTGG-3′ and 5′-TCCTCGAGCTAGATGGAGGCGGCGGACGGCTTCACCTTG-3′ for extracellular expression. The amplified fragment was digested at the *Aat*II and *Xho*I sites and inserted into the *Aat*II and *Xho*I sites of pWI3α (*15*). The resultant plasmid was named pWI3αSA.

Transformation. The plasmids pUI3αSAFlo318 and pWI3αSA were introduced into *S. cerevisiae* MT8-1 by the lithium acetate method using Yeastmaker (Clonetech Laboratories, Polo Alto, CA) according to the procedure recommended by the supplier.

Western Blot Analysis. After cultivation, the strains were collected by centrifugation. They were then washed twice with phosphate-buffered saline (PBS: 50 mM phosphate and 150 mM sodium chloride, pH 7.4). The cells, buffer, and glass beads (0.5 mm in diameter) were mixed at a ratio of 1:2:1 (wt/vol/wt) in a microcentrifugation tube and agitated vigorously with a vortex mixer. The supernatant (10 μL) obtained was applied on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE). The proteins separated on the gel were electroblotted on a poly(vinylidene difluoride) (PVDF) membrane and subsequently incubated with 10 μL of goat anti-streptavidin antibody (Vector Laboratory Inc., Burlingame, CA). Using 200 μL of 150 μg/mL rabbit anti-goat IgG (H+L) antibodies (IgG) conjugated with horseradish peroxidase (HRP) (Kirkegaard Perry Laboratories, Gaithersburg, MD), we detected transferred native and fused streptavidin subunits.

Fluorescence Microscopy. The active streptavidin on the cell surface was observed as described below. Cells were collected by centrifugation at 6000*g* for 5 min, washed with PBS, and adjusted to OD_{600} = 10 with PBS; 200 μL of the cell suspension was centrifuged in a 1.5 mL microtube. The collected cells were incubated with 200 μL of a 1:250 dilution of biotinylated fluorescein isothiocyanate (FITC) (Pierce, Rockford, IL) for 30 min at room temperature. After washing the cells with PBS, FITC-labeled yeast cells were observed by phase contrast and immunofluorescence microscopies.

ELISA Using *S. cerevisiae* Displaying Streptavidin. Aliquots of 10, 20, and 40 μL of the yeast cell suspension (OD_{600} = 20), prepared as described above, were centrifuged in 1.5 mL microtubes. The collected cells were resuspended in 200 μL of a series of diluted anti-streptavidin solutions (in PBS containing 1% BSA) and incubated for the desired time with rotation. The cells were then washed with PBS, resuspended in 200 μL of 150 μg/mL rabbit anti-goat IgG (H+L) antibodies (IgG) conjugated with HRP in PBS containing 1% BSA, and incubated for the desired time with rotation. Subsequently, the cells were washed twice with PBS and resuspended in 200 μL of PBS. Next, the HRP activity was measured using an HRP substrate kit (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and hydrogen peroxide (Bio-Rad Laboratories, Hercules, CA); 800 μL of the substrate was added to each amount of cell suspension, and the samples were incubated for the desired time. After removing the cells by centrifugation at 13 000 rpm for 10 s, the absorbance of the supernatant at 405 nm was measured.

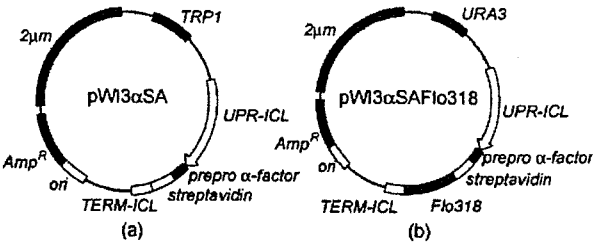

Figure 2. Plasmid vectors for extracellular expression of streptavidin (a, pWI3αSA) and cell surface display expression of streptavidin-Flo318 (b, pUI3αSAFlo318).

Results

Construction of Plasmids for Cell Surface Display and Extracellular Expression of Streptavidin and Confirmation of its Expression in Yeast. The plasmids pWI3αSA and pUI3αSAFlo318 (Figure 2) were constructed as described in Materials and Methods. As shown in Figure 2, pWI3αSA is for the extracellular expression of streptavidin, and pUI3αSAFlo318 is for the display of the fusion protein streptavidin-Flo318 on the cell surface. They are multicopy plasmids containing the prepro region of α factor as a secretion signal. These expressions are under the control of *UPR-ICL* (5′-upstream region of isocitrate lyase of *Candida tropicalis*) induced by glucose exhaustion (*16*, *17*). These plasmids were transformed into *S. cerevisiae* MT8-1, and three types of resultant transformant were obtained and named MT8-1/pUI3αSAFlo318 (SA-Flo), MT8-1/pWI3αSA (SA), and MT8-1/pUI3αSAFlo318/pWI3αSA (SA-Flo/SA).

S. cerevisiae MT8-1 cells (SA-Flo/SA, SA-Flo, and SA) were cultivated for 120 h in SDC medium as described in Materials and Methods. After cultivation, the expression of each protein was confirmed by Western blotting (Figure 3). Two bands corresponding to the native streptavidin and the fusion protein streptavidin-Flo318 were observed for the crude extract of the coexpressed strain (SA-Flo/SA, Figure 3, lane 1). On the other hand, single bands corresponding to the fusion protein streptavidin-Flo318 and native streptavidin separately expressed in the strains were detected (SA-Flo and SA, Figure 3, lanes 2 and 3). These findings indicate that the transformed genes of the native and fused streptavidins are expressed in each recombinant *S. cerevisiae* MT8-1.

Cell Surface Display of Streptavidin. To confirm the presence of the active streptavidin on the cell surface, the cultivated cells described above were incubated with biotinylated FITC. After washing with PBS, the biotinylated FITC-labeled cells were observed by fluorescence microscopy as described in Materials and Methods. As shown in Figure 4a, the coexpressing cells (SA-Flo/SA) were clearly labeled with

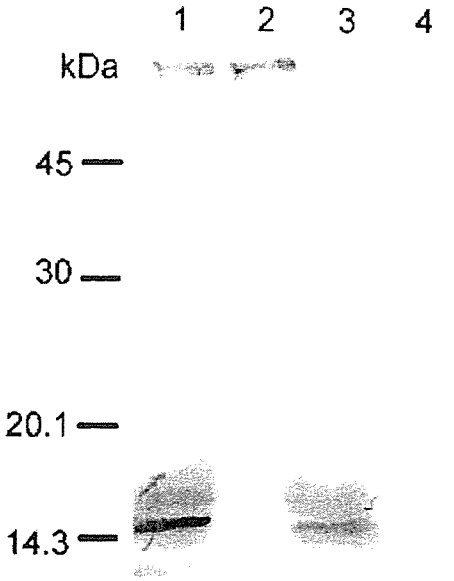

Figure 3. Immunoblotting of the extracts of cells expressing native and anchored subunits of streptavidin. Lane 1, SA-Flo/SA; lane 2, SA-Flo; lane 3, SA; lane 4, Flo. Lines show molecular weight markers.

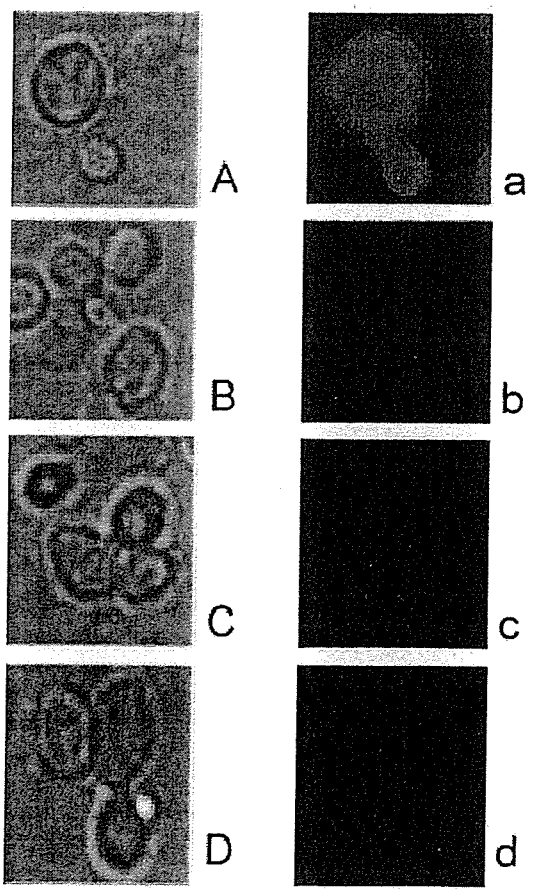

Figure 4. Microscopic observation of transformed cells labeled with biotinylated FITC. Phase contrast micrographs (left panels) and fluorescence micrographs (right panels) of yeast cells. (A and a) SA-Flo/SA; (B and b) SA-Flo; (C and c) SA; (D and d) Flo.

biotinylated FITC, whereas the other cells (SA-Flo, SA, and Flo) were not (Figure 4b–d). This suggests that the active streptavidin is successfully displayed on the coexpressing cell

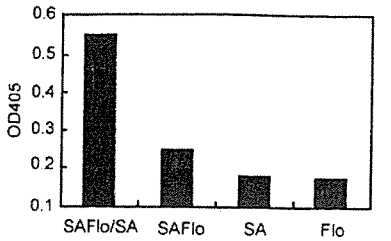

Figure 5. Activity of HRP adsorbed on each strain. Vertical axis indicates HRP activity by absorbance at 405 nm. The data points represent the average of three independent experiments.

surface (SA-Flo/SA). Although the two transformants (SA-Flo and SA) expressed each transformed gene, these proteins hardly assemble into an active form. On the other hand, when the anti-streptavidin antibody was used to detect both the active and inactive forms of streptavidin on the cell surface, the displayed streptavidins were detected not only on the coexpressing cells (SA-Flo/SA) but also on cells singly expressing the fusion protein streptavidin-Flo318 cells (SA-Flo) (data not shown). This suggests that the inactive streptavidin is displayed on cells singly expressing the fusion protein streptavidin-Flo318 cells (SA-Flo).

Adsorption of Biotinylated Horseradish Peroxidase. To quantitatively evaluate the amount of active streptavidin on the cell surface, biotinylated HRP was adsorbed onto the cultivated cells. After 10 min of incubation, the cells were washed and the HRP activity was measured (Figure 5). In this examination, only the coexpressing cells (SA-Flo/SA) bound biotinylated HRP. This also indicates that the active streptavidin is successfully displayed on the coexpressing cells (SA-Flo/SA). Although a small amount of active streptavidin was detected on the cells (SA-Flo), the active streptavidin might assemble into an active form incidentally in the cell wall.

Discussion

We have succeeded in the development of a yeast cell displaying active streptavidin, which is a homotetrameric protein, on the cell surface. This was achieved by the coexpression of the native and anchored subunits of streptavidin. Although the *S. cerevisiae* cells harboring only the plasmid pUI3αSAFlo318 (SA-Flo) expressed the heterologous protein (streptavidin-Flo), the expressed protein did not assemble into an active form. The C-terminal region Flo318, which is a serine/threonine-rich hydrophobic tail with the GPI anchor attachment signal for glycosylation, inhibited the correct assembly of the tetrameric streptavidin, whereas the active streptavidin was displayed on the coexpressing cells harboring the plasmids pUI3αSAFlo318 and pWI3αSA (SA-Flo/SA). In this system, the anchored subunit was probably immobilized on the cell surface first, and subsequently, the native subunit assembled with the immobilized subunit while being secreted extracellularly.

As shown in Figure 3, the amount of native streptavidin expressed by the coexpressing cells (SA-Flo/SA) was much larger than that of the singly expressing cells (SA). Inside the SA cells, a small amount of native subunit remained. On the other hand, the native subunit assembled with the immobilized subunit on the surfaces of the coexpressing cells (SA-Flo/SA). Further investigations on how to improve the promoter of pUI3αSAFlo318 for the constitutive production of the anchored subunit might lead to the development of a more effective expression system. Since streptavidin is one of the most useful tools for various biological experiments, attempts to develop whole-cell affinity adsorbents widely applicable to immunoas-

says and biosensing using biotinylated compounds are currently in progress. In this report, we confirm the display of active streptavidin on cell surface by the coexpression of the native and anchored subunits. To our knowledge, this is the first report concerning the display of a homo-oligomeric protein on a cell surface. This coexpression system is also expected to be applied in hetero-oligomeric proteins by fusing one subunit and a protein having an anchor region, such as α-agglutinin or Flo1.

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Secretory production system of bionanocapsules using a stably transfected insect cell line

Takuya Shishido · Masaru Muraoka · Masakazu Ueda · Masaharu Seno · Katsuyuki Tanizawa · Shun'ichi Kuroda · Hideki Fukuda · Akihiko Kondo

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Abstract Bionanocapsules (BNCs) are hollow nanoscale particles composed of L protein of the hepatitis B virus surface antigen that represent specific affinity for human hepatocytes. BNCs can transfer genes and drugs into human hepatocytes efficiently and specifically. BNC can be expressed in yeast cells. In this study, we developed a new L particle production system using a stably transfected insect cell line. For this purpose, we established a host–vector system using the *Trichoplusia ni* insect cell line. L particles were efficiently secreted by the overexpression of the L protein, which was fused to the secretion signal peptide. The concentration of L particles was reached approximately 1.7 μg/ml in 5 days during cultivation in a serum-free medium without antibiotic selective pressure. The production of L particles was maintained for at least 75 days. The secretory production of L particles facilitated their easy purification by chromatography. Furthermore, it was demonstrated that purified L particles can transfect only human hepatocytes. Therefore, an insect cell expression system is an attractive tool for the production of BNC.

T. Shishido · A. Kondo (✉)
Faculty of Engineering, Kobe University,
Kobe 657-8501, Japan
e-mail: akondo@kobe-u.ac.jp

M. Muraoka · H. Fukuda
Graduate School of Science and Technology, Kobe University,
Kobe 657-8501, Japan

M. Ueda
School of Medicine, Keio University,
Tokyo 160-0016, Japan

M. Seno
Graduate School of Natural Science and Technology,
Okayama University,
Okayama 700-8530, Japan

K. Tanizawa · S. Kuroda
The Institute of Scientific and Industrial Research,
Osaka University,
Ibaraki 567-0047, Japan

Introduction

Viral and nonviral gene transfer vectors were developed for gene therapy (El-Aneed 2004; Helge 2000; Schmidt-Wolf and Schmidt-Wolf 2003). Viral vectors, such as adenoviruses, adenoassociated viruses, and retroviruses, have some advantages such as high transduction efficiency and stability. However, there are safety concerns for the clinical applications of viral vectors (Schroder et al. 2002; Woods et al. 2003; Li et al. 2002). On the other hand, nonviral vectors, such as naked DNA and liposomes, are generally safe and do not have a limitation in the size of the transgene. However, the efficiency of gene transfer is low (Herweijer and Wolff 2003; Hirko et al. 2003). Furthermore, these vectors transfer genes to not only the target cells but also other cells. Thus, no system has so far fulfilled the required criteria of high cell and tissue specificity, high transgene efficiency, and high safety level.

We previously reported an efficient drug and gene delivery system with L particles derived from the hepatitis B virus (HBV) (Yamada et al. 2003; Yu et al. 2005). HBV is an enveloped DNA virus of Hepadnaviridae. The human hepatocytes infected with HBV synthesize and release not only 42 nm of HBV virions but also noninfectious 22 nm empty subviral particles, which are spherical and filamentous. The HBV genome encodes three envelope proteins: the S protein, which is a major constituent (226 amino acid residues) of the HBV envelope protein and empty HBV surface antigen (HBsAg) particles; the M protein, which

contains 55 additional amino acid residues (pre-S2 peptide) at the N terminus of the S protein; and the L protein, which contains the 108 (subtype *y*) or 119 (subtype *d*) additional amino acid residues (pre-S1 peptide) at the N terminus of the M protein (Heermann et al. 1984). L particles are hollow nanoparticles consisting of only the L protein and endoplasmic reticulum membrane-derived phospholipids (Yamada et al. 2001). L particles have several advantages compared with viral and nonviral vectors. Firstly, L particles show a high infectivity. Secondly, L particles are very safe because they are free from the viral genome. Thirdly, there is no practical limitation in the size of the transgene. Because of these reasons, the applicability of L particles can be extended to the delivery of proteins and chemical compounds. Furthermore, they have specificity for human hepatocytes. The results of in vitro and in vivo transfection experiments demonstrated that genes and drugs are specifically transferred into human hepatocytes using L particles (Yamada et al. 2003). It was suggested that L particles are strongly expected as potential candidates for the specific gene and drug delivery system.

To date, the preparation of HBsAg particles was studied in various expression systems, namely, yeast cells (Valenzuela et al. 1982), mammalian cells (Holzer et al. 2003), plant cells (Richter et al. 2000), and insect cells (Lanford et al. 1989; Hofmann et al. 1995; Deml et al. 1999a,b). We previously demonstrated the efficient production of L particles in recombinant yeast cells and 4 μg of the purified L particles was obtained from 1 ml of the culture medium after 7 days of cultivation (Yamada et al. 2001; Kuroda et al. 1992). However, L particles accumulate in the cytoplasm, therefore, yeast cell disruption and a subsequent purification step are required. On the other hand, the productivity of L particles is very low in mammalian cells (>50 ng/ml). Therefore, insect cells are desired as a new expression system for the production of L particles. A transient expression system using recombinant baculoviruses was reported previously (Lanford et al. 1989; Hofmann et al. 1995). However, insect cells are killed during infection cycle in this system and expressed recombinant proteins tend to be degraded by proteases. A stable expression system in stably transfected cell lines was also reported (Deml et al. 1999a,b). Although S particles or L particles coexpressed with the S protein are produced efficiently, an expression system with only L particles has not yet been established.

In this study, we constructed a new L particle expression system. The L protein was fused to the honeybee melittin secretion signal peptide and we examined the expression of the fused protein in insect cells. The stably transfected insect cell line can efficiently produce L particles and secrete into the culture medium. This production was stable for more than 2 months. Furthermore, it was indicated that the expressed L particles can be used for gene and drug delivery.

Materials and methods

Materials and strains

The *Escherichia coli* strain NovaBlue (*endA1 hsdR17* (rk12 −/mk12+) *supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proA+B* + *lacIqZ.M15* :: Tn*10* (TcR)]) (Novagen, Darmstadt, Germany) was used as a host for recombinant DNA manipulation. *E. coli* was cultivated in Luria–Bertani medium (1% tryptone, 0.5% yeast extract, and 1% NaCl; w/v) with 100 μg ampicillin/ml.

Cell culture

The *Trichoplusia ni* BTI-TN-5B1-4 insect cell line (High Five, Invitrogen, Carlsbad, CA, USA) was maintained in a serum-free medium (Express Five SFM, Invitrogen) supplemented with 0.26 g/l L-glutamine and 0.1% gentamicin (Invitrogen) at 27 °C. The human hepatocellular carcinoma cell line NuE was maintained in RPMI1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in 5% CO_2 (Murayama et al. 1999). The human hepatocellular carcinoma cell line HepG2 and human epidermal carcinoma cell line A431 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS.

Plasmids construction

The expression plasmid for the L protein was constructed by inserting a fragment of a large hepatitis B surface antigen (L protein, subtype *adr*) into pXINSECT-DEST38 (Invitrogen) and pIB/V5-His (Invitrogen). This insert was amplified from the plasmid pGLDLIIP39-RcT by polymerase chain reaction (Kuroda et al. 1992) using the forward primer 5′-GGGGGATCCACCATGAAATTCTTAGT CAACGTTGCCCTTGTTTTTATGGTCGTATA CATTTCTTACATCTATGCCATGGGGAC GAATCTTTCTGTTCCC-3′, including the honeybee melittin secretion signal peptide sequence or 5′-GGGGGATC CATGAAGTTATGCATATTACTGGC CGTCGTGGCCTTTGTTGGCCTCTCGCTC GGGATGGGGACGAATCTTTCTGTTCCCAAT-3′, including the *Drosophila* binding protein (Bip) secretion signal peptide sequence, and the reverse primer 5′-CCCGCGGCCGCGTCGACCAGCTTTAACGAACG CAG-3′. The amplified fragment was digested at the *Bam*HI and *Not*I restriction sites and ligated into plasmids pXINSECT-DEST38 and pIB/V5-His. The resulting plas-

mids were designated pX-Mel, pX-Bip, pIB-Mel, and pIB-Bip. To obtain a stable transformant, plasmid pBmA.neo (Invitrogen), which contains the neomycin resistant gene, was used simultaneously with the plasmid pX-Mel and pX-Bip.

Transfection and selection of insect cell line

For the transient expression of L particles, High Five cells were seeded on a 35-mm dish at a density of 2×10^5 cells/ml 24 h before transfection, and the cells were transfected with 1 μg of pX-Mel, pX-Bip, pIB-Mel, and pIB-Bip using FuGENE 6 (Roche, Basel, Switzerland). Three days posttransfection, culture supernatant was recovered and L particles were assayed using an IMx enzyme immunoassay (EIA) kit (Abbott Laboratories, Abbott Park, IL, USA), which can specifically detect the particulate forms of HBsAg but not the free HBsAg protein.

For the generation of stably transfected cell lines, High Five cells were cotransfected with pX-Mel and pBmA.neo or pX-Bip and pBmA.neo with weight ratios of 10:1, 50:1, and 100:1 (pX:pBmA.neo). At 48 h posttransfection, transfected cells were resuspended in 12 ml of a fresh culture medium and distributed on a 12-well plate. After 1 day, the medium was replaced with fresh Express Five SFM containing 1.0 mg/ml G418 (Invitrogen) for selection. The selective medium was replaced every 4 days, and stably transfected cell lines were isolated after 3 weeks of selection with a G418-containing medium. The concentration of L particles was assayed after 4 days of cultivation of cloned cells using an IMx EIA kit.

L particle production with stably transfected High Five cells

For the analysis of the stability of L particle expression, stably transfected High Five cells were seeded on a 12-well plate at a density of 1×10^5 cells/ml in 1 ml of a fresh medium and allowed to grow for 8 days. The concentration of L particles from the culture supernatant was assayed everyday using an IMx EIA kit. The production stability of stably transfected High Five cells was examined for 75 days. The stably transfected cells were subcultured and L particles produced were quantified at 5-day intervals.

Western blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of L particles in culture supernatants was performed using 10% gel. Western blot analysis was performed using an anti-S protein antibody prepared from an immunized mouse as the primary antibody and an alkaline phosphatase (AP)-conjugated anti-mouse IgG antibody (Promega Co., Madison, WI, USA) as the secondary antibody. The colorimetric detection of AP activity was performed using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (Promega Co., Madison, WI, USA).

Purification of L particles from stably transfected cell culture supernatants

The culture supernatant (150 ml) of stably transfected insect cells was collected and concentrated fourfold by ultrafiltration using a Vivaspin20 device (Sartorius AG, Goettingen, Germany) at 4 °C. After concentration, the supernatant was loaded on sulfate Cellulofine resin (Chisso, Tokyo, Japan) equilibrated in 0.01 M sodium phosphate (pH 7.2) containing 0.15 M NaCl in a disposable gravity column (Clontech Laboratories, CA, USA), and washed. L particles were recovered by elution with 0.01 M sodium phosphate (pH 7.2) containing 0.5 M NaCl.

In vitro transfection of calcein with L particles

Calcein (0.1 mM, bis[*N,N*-bis(carboxymethyl)aminomethyl]fluorescein); Dojindo, Kumamoto, Japan) was mixed with purified L particles (240 ng) in 200 μl of phosphate-buffered saline, and electroporated into the particles using a Gene pulser II electroporation system (BioRad Laboratories, Hercules, CA, USA) in a 2-mm-gap cuvette, typically at 200 V and 1,000 μF for about 20 ms. The electroporated L particles were centrifuged at 8,000 rpm for 1.5 min and 60 ng of L particles recovered from supernatant were added to 2×10^4 cells of HepG2, NuE, and A431. Fluorescence was observed under an 5-Pa laser-scanning microscope (LSM) (Carl Zeiss, Oberkochen, Germany) at 6 h posttransfection at 37 °C.

Results

Establishment of High Five expression system of L particles

To investigate the dependence of L-particle productivity on the expression cassette, the transient expression of L particles in High Five cells was performed using pX-Mel, pX-Bip, pIB-Mel, and pIB-Bip. The expression plasmid pX contains the silkmoth (*Bombyx mori*) cytoplasmic actin gene promoter and pIB contains the promoter derived from the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (*Op*MNPV) (Fig. 1). In addition, for the efficient secretion of L particles, two types of secretion signal peptide, namely, the honeybee melittin signal peptide sequence (Mel signal) and the *Drosophila* Bip signal sequence (Bip signal), were investigated. The concentration of secreted L particles from transfected cells was deter-

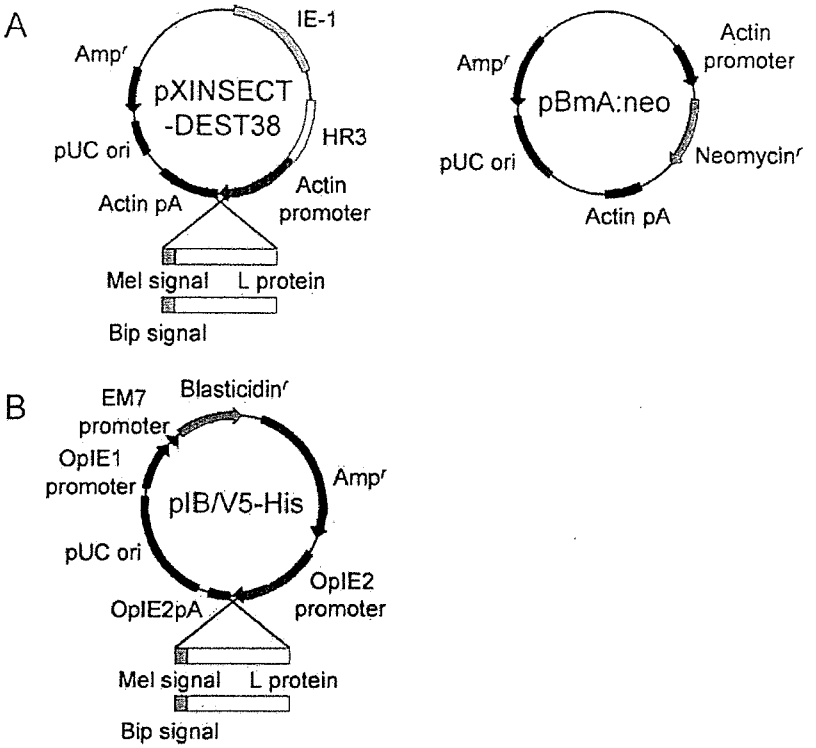

Fig. 1 Schematic representation of expression vector for L protein. One system based on pXINSECT-DEST38 including silkmoth (*Bombyx mori*) cytoplasmic actin gene promoter. In this system, pBmA.neo is used for antibiotic selection (**a**). Another system based on pIB/V5-His including promoter derived from baculovirus *Op*MNPV (**b**). The L protein was fused to the honeybee melittin secretion signal peptide and *Drosophila* Bip secretion signal peptide at its N terminus. Constructed L protein expression plasmids were named pX-Mel, pX-Bip, pIB-Mel, and pIB-Bip

mined 3 days after transfection using an IMx EIA kit (Fig. 2a). Results indicated that pX-Mel is more efficient than other expression plasmids. Furthermore, the results of Western blotting demonstrated that pX-Mel is the optimal expression plasmid for the secretory expression of L particles in High Five cells (Fig. 2b). Therefore, pX-Mel was used in the subsequent studies.

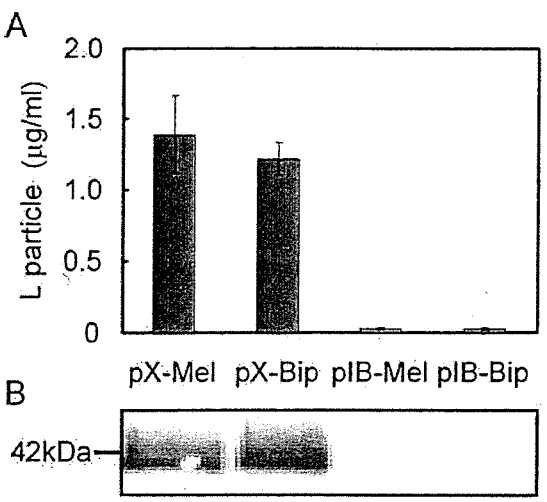

Fig. 2 Transient expression of L particles. pX-Mel, pX-Bip, pIB-Mel, and pIB-Bip were transfected into High Five cells using FuGENE 6. At 3 days after posttransfection, secreted L particles were quantified using an IMx EIA kit (**a**). The data points represent the average of three independent experiments. Western blot analysis was performed using the anti-S protein antibody (**b**)

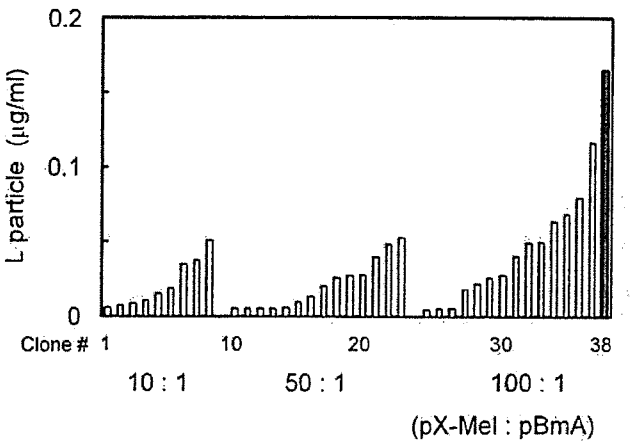

Fig. 3 Distribution of expression levels of L particles in stably transfected cell lines. High Five cells were cotransfected with pX-Mel or pX-Bip and pBmA.neo at the weight ratio of 10:1, 50:1, and 100:1 (expression plasmid pX:antibiotic resistance plasmid pBmA.neo). The amount of L particles was assayed after 3 days of subculture using an IMx EIA kit. The high-expression cell line #38 is represented by a *filled bar*

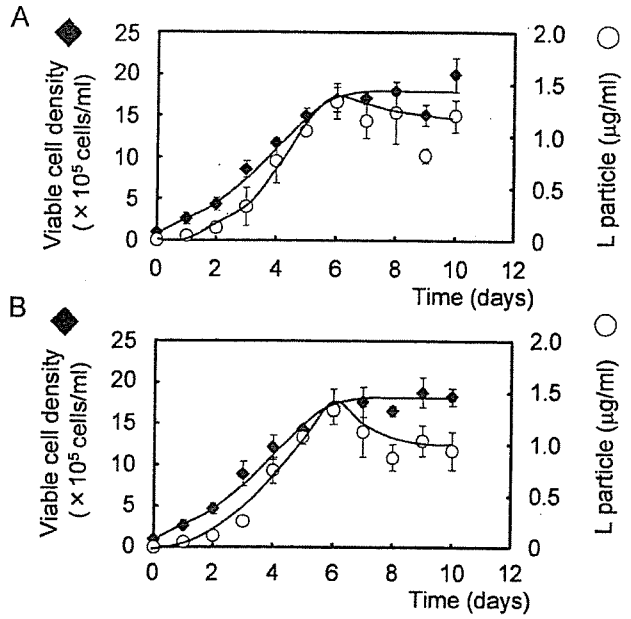

Fig. 4 Production of L particles in static culture of clone #38. Clone #38 was cultivated for 10 days with (**a**) and without G418 (**b**). The concentration of L particles in culture supernatant was determined using an IMx EIA kit. Viable cell density and L particle concentration are represented by *circles* and *diamonds*, respectively. The data points represent the average of three independent experiments

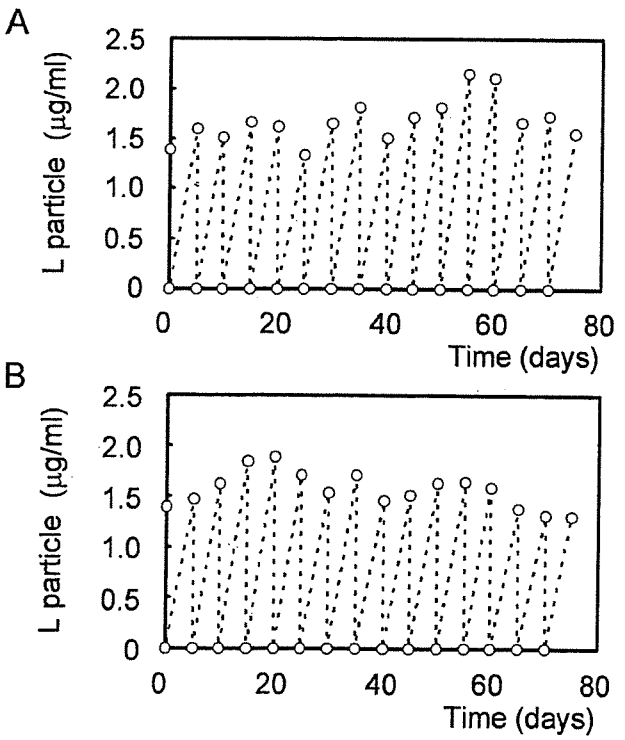

Fig. 5 Stability of L particle production in clone #38. The production stability of clone #38 was assessed every 5 days for 75 days with G418 (**a**) and without G418 (**b**)

Generation of stably transfected cell lines

High Five cells were cotransfected with pX-Mel and pBmA.neo as described in "Materials and methods." After antibiotic selection, 38 stably transfected clones were successfully obtained. Three days after the medium replacement in small static cultures, the concentration of L particles indicated the degree of heterogeneity of transformed cells (Fig. 3). The highest producer cell line (clone #38) was established by transfection at the weight ratio of 100:1 (pX:pBmA.neo). Therefore, clone #38 was used in the subsequent studies.

Production of L particle from clone #38 in static culture

Clone #38 was characterized in static batch cultures. The cells were seeded on a 12-well plate at a density of 1×10^5 cells in 1 ml of fresh medium in the presence or absence of antibiotic selective pressure (G418), and the concentration of L particles in culture supernatant was determined every day for 10 days using an IMx EIA kit. Clone #38 reached the maximum cell density of approximately 1.7×10^6 cells/ml and produced 1.3 μg/ml of L particles after 6 days of cultivation in the presence of G418 (Fig. 4a). A similar tendency was observed in the absence of G418 (Fig. 4b). These results indicated that clone #38 can produce L particles efficiently and should be subcultured to check the stability of clone #38 every 5 days at subconfluence (60–80%).

Stability of L particle production from clone #38

We confirmed the stability of clone #38, which represent high productivity of L particles in the presence or absence of antibiotic selective pressure in the long-term. The cells were subcultured every 5 days at an initial density of 1×10^5 cells/ml in a fresh medium and the culture medium was assayed for L particle production. The expression level of L particles did not change with time in the culture medium for 75 days in the presence or absence of 1.0 mg/ml G418 (Fig. 5). It is interesting to note that the average concentration of L particles reached approximately 1.7 μg/ml, which is higher than that in the static batch cultures (Fig. 4). Cell growth remained quite stable throughout the cultivation time. These results demonstrated that the stably transfected cell line showed an efficient production of L particles and the insect cell expression system was useful for vector production.

In vitro transfection of calcein with L particles

After purification by sulfate Cellulofine resin column chromatography, L particles were used for specific drug

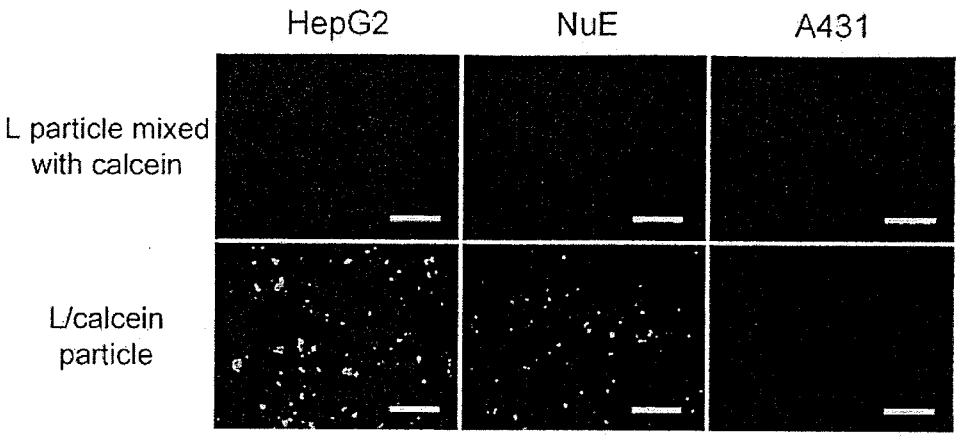

Fig. 6 Transfection of calcein with L particles. A mixture of L particles and calcein and calcein-carrying L particles (60 ng) were added to approximately 2×10^4 cells of HepG2, NuE, and A431. After 6 h of incubation at 37 °C, infection was evaluated by observing fluorescence under an LSM. *Scale bar*, 100 μm

delivery into hepatocytes. A fluorescent compound, calcein, was electroporated into L particles. The results showed that L particles can efficiently transfect the human hepatocarcinoma cell lines HepG2 and NuE (Fig. 6). On the other hand, fluorescence was not detected in the human epidermoid carcinoma cell line A431. Therefore, it was confirmed that L particles have specificity to hepatocyte and this result is consistent with the data for L particles obtained from yeast cells (Yamada et al. 2003).

Discussion

An insect cell expression system has several advantages in recombinant protein expression. The major advantage is a high level expression of secreted glycoproteins. Insect cell expression systems with HBsAg were demonstrated in previous reports. The expression of the S particle was investigated in the *Spodoptera frugiperda* Sf9 cell line using a baculovirus expression system (Lanford et al. 1989) and the stably transfected *Drosophila melanogaster* Schneider-2 (DS-2) cell line (Deml et al. 1999a,b). The coexpression of S and L proteins was achieved in the Sf9 cell line using a baculovirus expression system (Hofmann et al. 1995). However, an expression system with L particles consisting of only the L protein was not established to date except for yeast cell expression system.

We previously demonstrated that an L particle as a novel vector for the gene and drug delivery system can be prepared by overexpressing the L protein in *Saccharomyces cerevisiae* (Yamada et al. 2001; Kuroda et al. 1992). However, expressed L particles accumulate in the cytoplasm, therefore, complicated purification steps are required. Thus, we attempted to develop a stably transfected insect cell secretory expression system with L particles in this study.

To determine the host–vector system, two different systems were examined for transient expression using High Five cells, which are superior hosts for the production of recombinant proteins (Farrell et al. 1998; Keith et al. 1999). Moreover, the secretion signal peptide was fused to the L protein for the efficient production of L particles (Kuroda et al. 1992). These results indicated that the combination of pXINSECT-DEST38 and the melittin secretion signal peptide was suitable for the expression of L particles (Fig. 2). The difference in productivity between expression systems with various vectors might be attributed the nature of the promoter and the presence of homologous region 3 (HR3) and IE1 from *Bombyx mori* nuclear polyhedrosis virus in pXINSECT-DEST38 (Fig. 1a). HR3 is a viral transcriptional enhancer and it acts as a constitutive enhancer of the cytoplasmic actin promoter (Lu et al. 1997). IE1 is a viral transcriptional transactivator that stimulates L protein expression (Lu et al. 1996). Therefore, pXINSECT-DEST38 with the melittin secretion signal peptide represented high expression efficiency of L particles. The results of Western blot analysis suggested the homogeneous glycosylation of the L protein (Fig. 2b).

In this study, we successfully established the stably transfected insect cell lines for the production of L particles. The cotransfection of pX-Mel and pBmA.neo at the weight ratio of 100:1 (expression plasmid pX:antibiotic resistance plasmid pBmA.neo) produced higher L particle-expressing clones (Fig. 3). The concentration of expression vectors seemed to correlate with expression efficiency (Deml et al. 1999a). For practical applications, a long-term stability of expression of L particles is indispensable. Secretion stability was therefore continuously examined (Fig. 5). The result indicated that the expression level of L particles in clone #38 was stable for 75 days in the presence of G418 with an average concentration of approximately 1.7 μg/ml. The stable secretion of L particles for 75 days is considered to be sufficient for industrial applications. It is interesting to note that no significant difference in productivity was observed in the absence of G418. The nonrequirement of antibiotics will minimize the production cost of L particles.

The advantage of this stably transfected insect cell expression system is the secretory expression of L particles