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Characterization of bio-nanocapsule as a transfer vector targeting human hepatocyte carcinoma by disulfide linkage modification

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Abstract

The bio-nanocapsules (BNCs) composed of the recombinant envelope L-protein of hepatitis B virus constitute efficient delivery vectors specifically targeting human hepatocytes. Here, we have tried to enhance the stability of the BNCs because the L-proteins in the BNCs were aggregated due to random disulfide bridging when stored for a long period at 4 °C. The envelope protein contains fourteen cysteine residues in the S domain. Aggregation of the envelope proteins might be avoided if unessential cysteine residues are replaced or removed because the irreversible alkylation of the free sulfhydryl group protects against the aggregation and enhances the efficiency of encapsulation. In this study, the possibility of reducing the number of cysteine residues in the S domain to enhance the stability of the BNCs was assessed. The replacement of each cysteine residue by site-directed mutation showed that nine of fourteen cysteine residues were not essential to obtaining BNCs secreted into the culture media. Furthermore, upon evaluating the combination of these mutations, it was found that eight residues of replacement were acceptable. The mutant BNCs with replaced eight cysteine residues were not only more resistant against trypsin, but also more effective in transducing genes into human hepatoma-derived HepG2 cells than the original type BNC. Thus, we demonstrated that the minimized number of cysteine residues in the S domain could enhance the stability of the BNCs.

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Keywords: Bio-nanocapsule; HBV surface antigen; Delivery vector; Hepatocyte specific targeting; Enhanced stability

1. Introduction

A molecular targeting method that transfers drugs efficiently and safely in a cell type specific manner has been a subject of great attention in the development of a novel drug delivery system (DDS). In general, recent DDS technologies are based on carriers that are categorized as two independent methods, viral and non-viral. Each has its advantages and disadvantages. Viral DDS systems, which exploit viruses, such as retroviruses, adenovirus, adeno-associated virus and lentivirus, have been developed as gene transfer procedures for efficient transduction. However, the patients

Abbreviations: DDS, Drug delivery system; BNC, Bio-nanocapsule; DMEM, Dulbecco's modified Eagle medium; HBV, Hepatitis B virus; GFP, Green fluorescent protein; EIA, Enzyme immunoassay; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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would have to face the risks of not only having the viral genome integrated into their own genomes, but also of having genes transferred to unexpected cells or tissues in a non-specific manner, resulting in serious diseases, such as cancer [1,2]. A non-viral system, represented by cationic liposome or polyethylene glycol, is considered to be rather safer than a viral system. However, liposome as a transfer vector still needs improvement in order to confer as much efficiency as viral vectors, and specificity on the target cells or tissues [3]. The development of novel DDS vectors with the advantages of both vectors is eagerly awaited [4–6].

Over the last decade, recombinant envelope proteins have been developed as vaccines for hepatitis B virus (HBV) [7]. The envelope of HBV is composed mainly of three closely related surface proteins that are known as the large (L), the middle (M) and the small (S) proteins. They are encoded in one open reading frame of HBV genome translated from three different in-frame initiation sites. When these proteins were expressed, each S-, M- and L-protein was found to form hollow virus-like nanoparticles. The particle composed of L-protein showed, in particular, a specific affinity to human hepatocytes due to the hepatocyte recognition site localized in the amino terminus [8–12]. For the purpose of developing safe and efficient DDS vectors having a targeting potential for specific cells or tissues, we exploited the nanoparticles composed of recombinant envelope protein as bio-nanocapsule (BNC) prepared from mammalian cells or yeast cells. The BNC composed of L-protein (L-BNC) is a sphere that has an average diameter of 80 nm, which has been observed under atomic force microscope of about 110 molecules of L-protein [13]. This nanoparticle formation is also confirmed under thin section electron microscope [14]. We found that the L-BNC was extremely useful as a vector to deliver genes and pharmaceutical drugs to the human liver in vivo cells, as well as in vitro [15].

However, we experienced aggregation of envelope proteins in the BNCs during long-term storage. We report here that the aggregation is due to the false disulfide bridges and that replacement of cysteine residues in the L-protein enhances the stability of the BNCs.

2. Materials and methods

2.1. Cell culture

COS7 cells were maintained at 37 °C under 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 0.1% sodium bicarbonate. HepG2 cells and WiDr cells were maintained at the same conditions, but with 10% FBS.

2.2. Preparation of the L-BNCs in yeast cells

The L-BNCs were produced in *Saccharomyces cerevisiae* AH22^{-} transformed with the L-protein expression plasmid pGLDLIIP39-RcT and purified as previously described [16].

2.3. Alkylation of L-BNCs with iodomonoacetic acid (IAA)

During purification of L-BNC, IAA was added to the yeast extracts to modify free SH-groups, thereby preventing them from forming false disulfide bridges. IAA was added to 10 mM 15 min before the precipitation of L-BNC with PEG6000 so that IAA could be removed after reaction.

2.4. Comparison of calcein encapsulation into L-BNC with/without IAA modification

Calcein (Dojindo, Japan) was mixed at 1 mM with 240 μg of L-BNCs with or without IAA modification in 500 μl of PBS. This was followed by electroporation with a Gene Pulser II electroporation system (Bio-Rad, VA) in a 4-mm cuvette, at 50 V and 750 μF. After removal of free calcein by gel filtration, calcein incorporated into BNCs was measured by a fluorescence image analyzer LAS-1000 (Fujifilm, Japan).

2.5. Expression of the mutant L-BNCs

The DNA fragment coding of the HBV L-protein that was fused to the secretion signal of chicken lysozyme was inserted downstream of SR alpha promoter to construct the expression vector pBO441. To replace the cysteine residues with serine or alanine residues, the gene for the L-protein was subjected to site-directed mutagenesis. Each resulting plasmid was introduced into COS7 cells by a Gene Pulser II. After 15 h, the medium was replaced by CHO-SFM II (Invitrogen, CA). Four days after transfection, the conditioned medium was collected and concentrated by a Vivaspin 20 (MWCO 1000 kDa, Vivascience, Germany). The BNCs and the derivatives in the conditioned media were immunologically detected and quantified with the Abbott IMx HBsAg assay system (Abbott Laboratories, IL) in the series of two-fold dilution. All the tests were performed and interpreted in accordance with the manufacturer's recommendation. Standard curve of EIA was evaluated with the positive control of HBsAg supplied with the system and antigen in the range of 10–100 ng/ml was estimated. Each sample was measured in triplicates and standard deviation was calculated.

3. Detection of the L-protein and its derivatives by Western blotting

The BNCs in the conditioned media were immunoprecipitated with anti-S antibody conjugated to microparticles, a component of the Abbott IMx HBsAg assay system, and then subjected to SDS-PAGE and Western blotting. The blots were probed with anti-S goat IgG conjugated to biotin and anti-biotin rabbit IgG conjugated to alkaline phosphatase. Immunoreactive bands were detected with the Phototope-Star Chemiluminescent Detection Kit (New England Biolabs, MA). The bands detected were densitometorically analyzed using NIH Image.

3.1. Protease protection assay

The BNCs were immunoprecipitated by anti-S antibody, and treated with various concentration of trypsin for an hour at 37 °C. The digestion fragments were detected using Western blotting, as described above.

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