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ORIGINAL ARTICLE

Delivery of chimeric hepatitis B core particles into liver cells

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Abstract

Aims: To display a liver-specific ligand on the hepatitis B virus core particles for cell-targeting delivery.
Methods and Results: A liver cell–binding ligand (preS1) was fused at the N-terminal end of the hepatitis B core antigen (HBcAg), but the fusion protein (preS1His_6HBcAg) was insoluble in *Escherichia coli* and did not form virus-like particles (VLPs). A method to display the preS1 on the HBcAg particle was established by incorporating an appropriate molar ratio of the truncated HBcAg (tHBcAg) to the preS1His_6HBcAg. Gold immunomicroscopy showed that the subunit mixture reassembled into icosahedral particles, displaying the preS1 ligand on the surface of VLPs. Fluorescence microscopy revealed that the preS1 ligand delivered the fluorescein-labelled VLPs into the HepG2 cells efficiently.
Conclusions: Chimeric VLPs containing the insoluble preS1His_6HBcAg and highly soluble tHBcAg were produced by a novel incorporation method. The preS1 ligand was exposed on the surface of the VLPs and was shown to deliver fluorescein molecules into liver cells.
Significance and Impact of Study: The newly established incorporation method can be used in the development of chimeric VLPs that could serve as potential nanovehicles to target various cells specifically by substituting the preS1 ligand with different cell-specific ligands.

Introduction

Hepatitis B virus (HBV) is one of the smallest human pathogenic DNA viruses, which belongs to the *Hepadnaviridae* family. HBV infects hepatocytes specifically and causes acute and chronic liver diseases in human. The virus is enveloped by a lipid membrane containing three related surface antigens (HBsAg) known as large (L-), middle (M-) and small (S-) HBsAg, which have different N-terminal extensions. The L-HBsAg consists of the N-terminal preS1, followed by the preS2 and the C-terminal S regions. The M-HBsAg and S-HBsAg are lacking the preS1 and the whole preS (preS1 + preS2) regions, respectively (Ganem 1991). The preS1 region carries all information needed for host specificity and tissue tropism (Glebe and Urban 2007). This region is responsible for both the initial attachment and specific receptor recognition of the virus (Glebe and Urban 2007; Le Duff *et al.* 2009). The L-HBsAg particles produced in yeast cells and mammalian cells have been shown to deliver green fluorescent protein (GFP; Yu *et al.* 2005) and plasmids carrying the GFP gene (Yamada *et al.* 2003; Iwasaki *et al.* 2007) into hepatocytes. Recently, Tang *et al.* (2009) demonstrated that T7 phage displaying the preS1 region delivered the phage and its gene specifically into HepG2 cells. This finding supports the potential usage of the preS1 region as a ligand to target hepatocytes.

HBV core (HBcAg) particle is a well-characterized VLP for its self-assembly property and its ability to display foreign epitopes in the development of multicomponent

vaccines. Many studies have demonstrated the insertion of foreign epitopes at the N-terminal end and the immunodominant regions of HBcAg, which resulted in the surface display of the foreign inserts on the VLPs (Kratz *et al.* 1999; Vogel *et al.* 2005; Skamel *et al.* 2006; Tan *et al.* 2007; Nassal *et al.* 2008; Yap *et al.* 2009). Recently, interest has been focused on the employment of the HBcAg particles as a gene delivery system. Cooper and Shaul (2005, 2006) demonstrated the unspecific delivery of oligonucleotides using the particles formed by the full-length HBcAg via the clathrin-mediated endocytosis pathway. In addition, a method to package GFP molecules was established recently by the VLPs formed by HBcAg (Lee and Tan 2008). These lay the foundation for exploring the potential application of the HBcAg particle as a nanocontainer to capture therapeutic molecules such as genes and drugs for specific targeted delivery into cells.

In this study, the preS1 region fused at the N-terminal end of the HBcAg was employed as a liver cell-targeting ligand. The fusion protein, namely preS1His_6HBcAg, was expressed abundantly in *E. coli* but was insoluble and did not assemble into VLPs. However, a method to reconstruct soluble chimeric VLPs displaying the preS1 ligand in the presence of truncated HBcAg (tHBcAg) was established. In this study, about 36% of the recombinant preS1His_6HBcAg subunit coassembled with the tHBcAg and gave rise to soluble VLPs. The preS1 ligand displayed on the VLPs was found to be functionally active and was able to translocate into HepG2 cells with high efficiency.

Materials and methods

Construction of recombinant plasmids encoding the preS1His_6HBcAg

The coding sequence of His_6-β-L-HBcAg (Fig. 1b) was amplified from the plasmid pHis-β-L-HBcAg (Yap *et al.* 2009) using the DyNazyme™ EXT DNA polymerase (Finnzymes, Vantaa, Finland; 1 U) in the presence of 0·2 mmol l^{-1} dNTPs, 0·1 ng template, 0·5 μmol l^{-1} forward primer (5′-AAA CCA T**GG TAC C**AG TCG ACA TGC GGG GTT CTC ATC ATC A-3′) and 0·5 μmol l^{-1} reverse primer (5′-TCC TCA AAC AGA AGC TTC CGG AAG CGT TGA TAG GAT AGG-3′). The underlined nucleotide sequences are the cutting sites of *Nco*I and *Hind*III, respectively. The bolded nucleotide sequence is the extra cutting site for *Kpn*I inserted at the 5′ end of His_6-β-L-HBcAg gene for further direct cloning purpose. The reaction conditions [initial denaturation (94°C, 2 min); denaturation (94°C, 10 s); annealing (62·7°C, 30 s); extension (72°C, 30 s)] for PCR amplification were set and performed in a thermocycler (Mastercycler

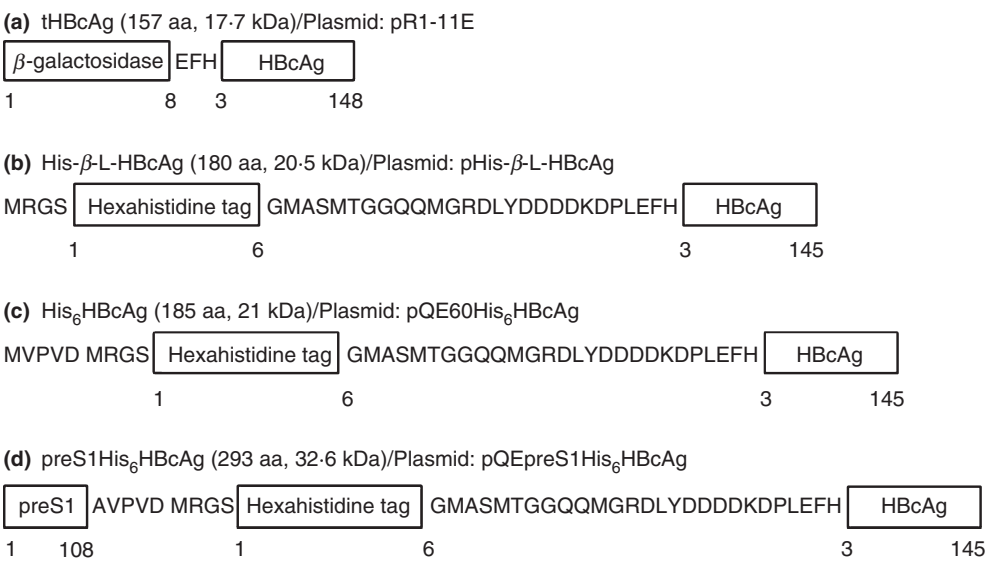

Figure 1 Primary structures of the tHBcAg and its fusion proteins. Schematic representation of the primary structures of the recombinant proteins, (a) tHBcAg, (b) His-β-L-HBcAg, (c) His_6HBcAg and (d) preS1His_6HBcAg. The hexahistidine tag for all His-β-L-HBcAg, His_6HBcAg and preS1His_6HBcAg is separated from the HBcAg by a linker (27 residues) containing the translated product of the T7 gene 10 sequence, the Xpress™ epitope tag and the enterokinase recognition site from the vector, pRSET B. The MVPVD residues at the N-terminal end of His_6HBcAg are the amino acids translated from the insertion of *Nco*I, *Kpn*I and *Sal*I cutting sites at the 5′ end of the His-β-L-HBcAg gene. Insertion of these residues does not affect the formation of VLPs. The preS1 ligand is inserted directly at the N-terminal end of the His_6HBcAg. The names of the respective proteins and plasmids are shown on top of the primary structures. Numbers in parentheses indicate the total number of amino acids and the predicted molecular mass (kDa) of the proteins.

personal; Eppendorf, New York, NY, USA) for 30 cycles and a final extension at 72°C, 30 min. The PCR product and pQE-60 vector (Qiagen, Valencia, CA, USA) were digested with *Nco*I and *Hin*dIII. The digested PCR product was then ligated to the linearized pQE-60 vector, using 1 U of T4 DNA ligase (Promega, Madison, WI, USA) at 4°C, overnight. The ligation mixture containing the recombinant plasmid, pQE60His$_6$HBcAg, was then introduced into *E. coli* strain M15 (pREP4) competent cells.

To fuse the preS1 ligand to the N-terminal end of the His$_6$HBcAg (Fig. 1c), the coding sequence of HBV preS1$_{1\text{-}108}$ (subtype *adyw*) was amplified with PCR as mentioned previously from the plasmid pQ264 (Tan *et al.* 1999) by the forward (5′-AAA CCA TGG GGC AGA ATC TTT CCA C-3′) and reverse (5′-TTT GGT ACC GCG GCC TGA GGA TGA GTG GTT C-3′) primers. The underlined nucleotide sequences indicate the *Nco*I and *Kpn*I cutting sites, respectively. PCR conditions were set as described earlier with a lower annealing temperature (60·4°C). The PCR product was inserted into plasmid pQE60His$_6$HBcAg after being digested with *Nco*I and *Kpn*I restriction endonucleases. The recombinant plasmids, pQE60His$_6$HBcAg and pQEpreS1His$_6$HBcAg, were verified by restriction endonuclease digestions, and the nucleotide sequences of the inserts were confirmed by DNA sequencing.

Expression and purification of recombinant proteins

Truncated HBcAg (residues 3–148; tHBcAg; Fig. 1a) was produced in *E. coli* strain W3110IQ carrying pR1-11E plasmid as described in the study by Lee and Tan (2008). The tHBcAg was purified by sucrose density gradient ultracentrifugation as described in the study by Lee and Tan (2008).

For the expression of preS1His$_6$HBcAg (Fig. 1d), *E. coli* cells harbouring the recombinant plasmid pQE-preS1His$_6$HBcAg was grown in Luria–Bertani (LB) broth (pH 7) containing ampicillin (100 μg ml^{-1}) and kanamycin (30 μg ml^{-1}). Protein expression was induced by adding 1 mmol l^{-1} isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 5 h. The recombinant protein was purified using the Ni^{2+} affinity HisTrap^{TM} FF prepacked column (1 ml; Amersham Biosciences, Buckinghamshire, UK). Purification was performed as recommended by the manufacturer with some modifications. The cells were harvested at 4°C, 5000 ***g*** for 10 min. The supernatant was discarded, and the cell pellet was resuspended in lysis buffer [50 mmol l^{-1} Tris (pH 8·0), 0·1% (v/v) Triton X-100, 0·2 mg ml^{-1} lysozyme, 4 mmol l^{-1} $MgCl_2$ and 7·5 μg ml^{-1} DNase I]. The suspension was incubated at 25°C for 2 h with gentle agitation. After that, the insoluble preS1His$_6$HBcAg together with bacteria cell debris was collected by centrifugation at 4°C, 6700 ***g***, for 30 min. The preS1His$_6$HBcAg was solubilized by resuspending the pellet in binding buffer (50 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl, 2·5 mol l^{-1} urea and 20 mmol l^{-1} imidazole; pH 7·4), sonicated for 1 min on ice with 10-s intervals between pulses and clarified by centrifugation at 4°C, 12 000 ***g*** for 20 min. The clarified supernatant was applied to a HisTrap$^{™}$ FF column and washed with 20 column volumes (CV) of binding buffer. The target protein was eluted (1 ml per fraction) with 5 CVs of elution buffer (50 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl, 2·5 mol l^{-1} urea and 500 mmol l^{-1} imidazole, pH 7·4). The purified protein was analysed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and its purity was about 90%. Protein concentration was determined as described by Bradford (1976).

Incorporation of the preS1His$_6$HBcAg into tHBcAg particles

To incorporate the preS1His$_6$HBcAg into the tHBcAg particles, three different molar ratios of the tHBcAg particles to the preS1His$_6$HBcAg (4 : 1, 2 : 1, 1 : 1) were prepared and dissociated together in the presence of 2·5 mol l^{-1} urea as described by Lee and Tan (2008). In brief, the dissociation was achieved by incubating 5 mg of tHBcAg particles together with the purified preS1His$_6$HBcAg, according to the protein ratio as mentioned earlier, in the presence of 2·5 mol l^{-1} urea at 25°C for 3 h. The dissociation step was performed in a final volume of 20 ml. For the assembly of chimeric particles (preS1His$_6$HBcAg + tHBcAg), the urea content was removed from the protein mixtures via dialysis in dialysis buffer [50 mmol l^{-1} Tris–HCl (pH 8·0) and 150 mmol l^{-1} NaCl; 1 l, three times] at 4°C. The dialysed samples were then concentrated with a 300-kDa cut-off polyethersulfone membrane VIVASPIN 6 (Sartorius Stedim Biotech, Aubagne Cedex, France) and fractionated on 8–40% (w/v) sucrose gradient at 4°C, 210 000 ***g***, for 5 h. The fractions were collected (1 ml per fraction) from the top of the tube and analysed using SDS-PAGE. In this experiment, preS1His$_6$HBcAg (5 mg) and tHBcAg particles (5 mg) treated individually with the same condition (as mentioned earlier) were used as controls.

To determine the formation of chimeric particles (preS1His$_6$HBcAg + tHBcAg) and the display of preS1 region on the surface of the particles, protein fractions containing the chimeric particles were pooled and dialysed against dialysis buffer (1 l, three times). The protein samples were concentrated to appropriate concentration and analysed using SDS-PAGE, Western blotting,

enzyme-linked immunosorbent assay (ELISA), transmission electron microscopy (TEM) and colloidal gold immunoelectron microscopy. The percentage of the preS1His_6HBcAg incorporated into the chimeric particles was determined by analysing the relative intensity of the protein bands for both the preS1His_6HBcAg and the tHBcAg on a Coomassie Brilliant Blue (CBB)-stained gel, using the GelDoc 2000 imaging system (Bio-Rad, Philadelphia, PA, USA), as described by Ng *et al.* (2006).

Western blot analysis

The proteins in the polyacrylamide gels were transferred onto nitrocellulose membranes using the Trans-blot®SD Semi-dry Transfer System (Bio-Rad, Canada, USA). The membranes were blocked with milk diluents (1 : 10 dilutions; KPL, Gaithersburg, MD, USA) at room temperature (RT) for 1 h. Primary antibody [guinea pig anti-HBsAg polyclonal antibody (pAb), subtype *ad*, 1 : 2500 dilutions (MP Biomedicals, Solon, OH, USA); or mouse anti-His monoclonal antibody (mAb), 1 : 5000 dilutions (Amersham Biosciences, Solon, OH, USA); or rabbit anti-HBcAg serum, subtype *adyw*, 1 : 10 000 dilutions] was added and incubated at RT for 1 h. The membranes were then washed three times (10 min each) with TBST [50 mmol l^{-1} Tris–HCl; pH 7·6, 150 mmol l^{-1} NaCl and 0·05% (v/v) Tween 20] prior to incubation with the secondary antibody conjugated to alkaline phosphatase (anti-guinea pig mAb, or anti-mouse mAb, or anti-rabbit mAb; 1 : 5000 dilutions; KPL), accordingly. The blots were washed for another three times (10 min each) with TBST, and colour was developed by adding substrates, 5-bromo-4-chloro-3′-indolyl phosphate (BCIP; Fermentas, York, UK) and nitro blue tetrazolium chloride (NBT; Fermentas, York, UK).

Enzyme-linked immunosorbent assay

The purified chimeric particles (preS1His_6HBcAg + tHBcAg) were first coated on a microtitre plate (0–500 ng, 100 μl per well) at 4°C for 16 h. After that, the coated wells were washed with TBST buffer and blocked with milk diluents (1 : 10 dilution, 250 μl per well) at RT for 1 h. The wells were then washed with TBST buffer, and the primary antibody (guinea pig anti-HBsAg pAb, subtype *ad*, 1 : 2500 dilutions; or mouse anti-His mAb, 1 : 5000 dilutions; or rabbit anti-HBcAg serum, subtype *adyw*, 1 : 10 000 dilutions) was added to the wells (200 μl per well). After 1-h incubation at RT, the wells were washed three times (10 min each) and incubated with the secondary antibody (anti-guinea pig mAb or anti-mouse mAb or anti-rabbit mAb; 1 : 5000 dilutions; 200 μl per well), accordingly, at RT for 1 h. The wells were then washed with TBST (three times, 10 min each). Substrate solution [0·001% (w/v) para-nitrophenyl phosphate, 9·55% (v/v) diethanolamine and 0·5 mmol l^{-1} $MgCl_2$; pH 9·8; 200 μl] was added and incubated at RT for colour development. The reaction was terminated by adding 3 mol l^{-1} NaOH (50 μl per well), and the absorbance at 405 nm (A_{405}) was measured using a microtitre plate reader (Bio-Tek, Winooski, VT, USA).

Transmission electron microscopy

Protein samples (≈0·3 mg ml^{-1}; 15 μl) were absorbed onto carbon-coated grids (200 mesh) and stained with uranyl acetate [2% (w/v)] as described by Lee and Tan (2008). The grids were observed under a TEM (HMG 400; Philips, Amsterdam, Netherlands).

Colloidal gold immunoelectron microscopy

The preparation for colloidal gold immunoelectron microscopy was performed as described by Kho *et al.* (2001) with some modifications. Briefly, the purified chimeric particles (≈0·5 mg ml^{-1}) were absorbed onto carbon-coated grids for 10 min. Later, the samples were probed with either anti-HBsAg pAb or anti-His mAb (1 : 50 dilution) in blocking buffer [50 mmol l^{-1} Tris–HCl (pH 7·4), 150 mmol l^{-1} NaCl and 1% (w/v) bovine serum albumin (BSA)] for 1 h. The samples were then washed four times with the same buffer (1 min each) and incubated with ImmunO™ protein A (5-nm gold conjugate, MP Biomedicals, Illkirch, France) diluted 1 : 50 in blocking buffer for another 1 h. The grid was then washed as mentioned earlier, rinsed three times with distilled water (dH_2O) and stained with uranyl acetate for 5 min. The grids were observed under a TEM (Philips HMG 400). In this experiment, the purified tHBcAg particles were used as negative controls.

Fluorescein labelling of the truncated HBcAg

The tHBcAg was labelled at the amino groups of lysine residues at positions 7 (K_7) and 96 (K_{96}) using the amine-reactive NHS-fluorescein reagent (Pierce, Rockford, IL, USA). The labelling procedure was performed as recommended by the manufacturer with some modifications. In brief, the NHS-fluorescein (15 molar in excess) was dissolved in dimethylsulfoxide (DMSO) and incubated with the purified tHBcAg [5 mg ml^{-1} in PBS (pH 7·4)] for 16 h at 4°C to produce fluorescein-labelled tHBcAg (FtHBcAg) particles. The nonreacted NHS-fluorescein was removed by dialysis against PBS (pH 7·4; 1 l, six times) at 4°C.

Incorporation of preS1His$_6$HBcAg into fluorescein-labelled tHBcAg (FtHBcAg) particles

To determine the translocation activity of the preS1 ligand displayed on the surface of the chimeric particles (preS1His$_6$HBcAg + tHBcAg), the nonfluorescent preS1His$_6$HBcAg was later incorporated into the dissociated FtHBcAg particles in the presence of 2·5 mol l^{-1} urea. The incorporation was performed as mentioned earlier by substituting the tHBcAg particles with 5 mg FtHBcAg particles (FtHBcAg:preSiHis6HBcAg ratio, 4 : 1). The reassembled fluorescent chimeric particles (preS1His$_6$HBcAg + FtHBcAg) were concentrated with a 300-kDa cut-off polyethersulfone membrane (VIVASPIN 6) and purified by sucrose density gradient (8–40% w/v) ultracentrifugation at 210 000 ***g*** at 4°C for 5 h as described by Lee and Tan (2008). The fractions containing the fluorescent chimeric particles were pooled and analysed using SDS-PAGE. The particle formation was confirmed by TEM.

Fluorescence microscopy

HepG2 cells were used as a liver cell model for the cell translocation study. HeLa cells were used as negative control. The cells were seeded (800 000 cells per well) onto sterile glass coverslips in a six-well plate (TPP, USA) and grown in DMEM medium (Sigma, St Louis, MO, USA) containing 10% (v/v) foetal bovine serum (FBS; Sigma) at 37°C in a humidified atmosphere containing 5% CO_2. The purified fluorescent chimeric particles (25 μg) were added to the cells in a total volume of 2-ml medium and incubated for 8 and 16 h, separately. After washing six times with PBS (pH 7·4), the cells were fixed with 3·7% (w/v) paraformaldehyde (prepared in PBS, pH 7·4) at 25°C for 10 min. The fixed cells were then mounted onto a drop of mounting medium [90% (v/v) glycerol, 20 mmol l^{-1} Tris–HCl (pH 8·5) and 0·1 mol l^{-1} propyl gallate] on a glass slide, sealed with nail polish and observed under a fluorescence microscope. In this experiment, the purified FtHBcAg particles served as a negative control.

Immunofluorescence microscopy

To prove the translocation of chimeric particles into HepG2 cells, the internalized tHBcAg was detected using anti-HBcAg serum. The preS1His$_6$HBcAg + tHBcAg chimeric particles (25 μg) were added to HepG2 cells and incubated for 16 h as mentioned earlier. After washing, the cells were fixed with 3·7% (w/v) paraformaldehyde (prepared in PBS, pH 7·4) for 10 min at 25°C and permeabilized immediately with ice-cold methanol for 6 min at −20°C. The permeabilized cells were incubated with rabbit anti-HBcAg serum (1 : 200 dilutions in PBS containing 0·2 mg ml^{-1} BSA, 1 h, 25°C), followed by adding the DyLight™ 488 conjugated donkey anti-rabbit pAb (1 : 100 dilutions in PBS containing 0·2 mg ml^{-1} BSA, 1 h, 25°C; BioLegend®, San Diego, CA, USA) in the dark. The cells were washed and mounted onto a drop of mounting medium on a glass slide, sealed with nail polish and observed under a fluorescence microscope. In this

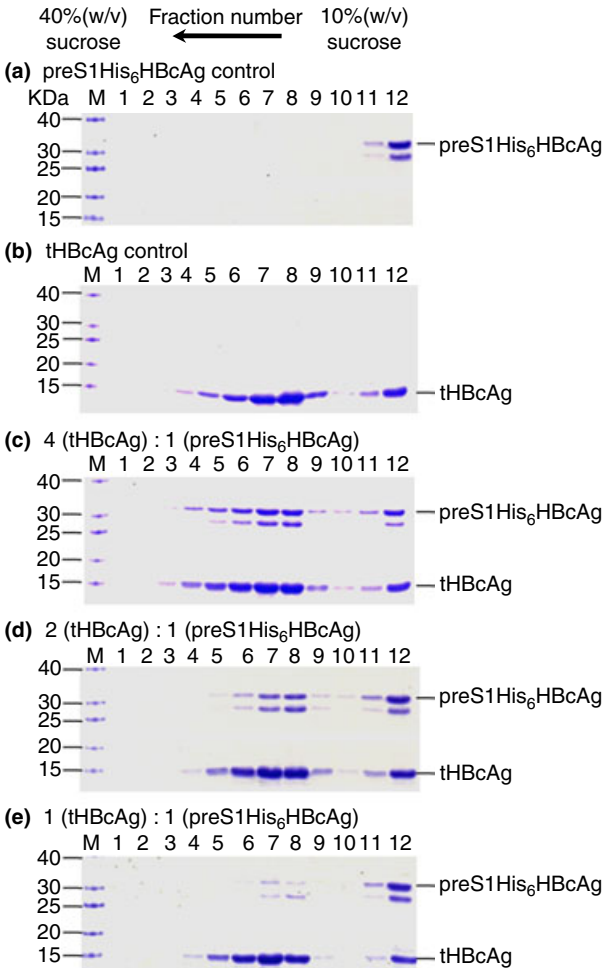

Figure 2 Incorporation of the preS1His$_6$HBcAg into the tHBcAg particles. Different molar ratios of the tHBcAg to preS1His$_6$HBcAg [4 : 1 (c), 2 : 1 (d) and 1 : 1(e)] were prepared and incubated at 25°C in the presence of 2.5 mol l^{-1} urea for 3 h. The chemical was removed by dialysis, and the protein mixtures were concentrated and fractionated on an 8–40% (w/v) sucrose gradient. Fractions (1 ml) were collected and analysed using SDS-PAGE. (a) and (b) are the preS1His$_6$HBcAg and tHBcAg controls, respectively. The CBB-stained gel shows a conspicuous band of tHBcAg (≈17 kDa) in fractions 4–9 (b)-(e). A band of approximately 33 kDa shows the comigration of the incorporated preS1His$_6$HBcAg into the tHBcAg particles (c)-(e). Excessive preS1His$_6$HBcAg applied to the incorporation process was observed at the top of the sucrose gradients. The apparent molecular masses (kDa) of the protein markers (lane M) are indicated on the left.

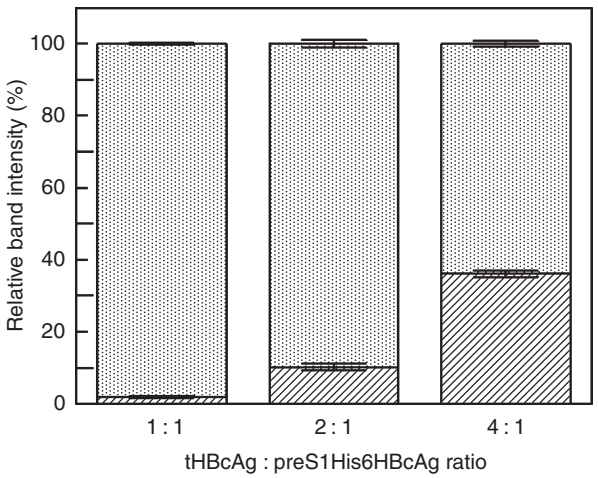

Figure 3 Incorporation level of the preS1His$_6$HBcAg in the reconstructed preS1His$_6$HBcAg + tHBcAg chimeric VLPs. Protein mixtures with different molar ratios of tHBcAg to preS1His$_6$HBcAg were prepared and purified on 8–40% (w/v) sucrose gradients. Fractions containing the preS1His$_6$HBcAg + tHBcAg chimeric particles were pooled and concentrated to appropriate protein concentration. Relative intensity of protein bands, tHBcAg (⬚) and preS1His$_6$HBcAg (▨), was determined by analysing the CBB-stained SDS-PAGE using the GelDoc 2000 imaging system; 36.1 ± 0.8% of the preS1His$_6$HBcAg was incorporated into the tHBcAg particles for the ratio 4 : 1 of tHBcAg to preS1His$_6$HBcAg. The level of incorporation decreased to 10.4 ± 1.0% and 2.0 ± 0.3% for ratios 2 : 1 and 1 : 1, respectively. Data points are mean ± standard deviations of triplicate determinations.

experiment, the nonpermeabilized cells treated with the same condition served as a negative control.

Results

Construction and expression of the preS1His$_6$HBcAg

To construct the recombinant plasmid, pQEpreS1His$_6$HBcAg, which encodes the preS1His$_6$HBcAg, the coding sequence of preS1$_{1\text{-}108}$ was amplified with PCR and inserted into plasmid pQE60His$_6$HBcAg. Figure 1 depicts the primary structures of tHBcAg, His-β-L-HBcAg, His$_6$HBcAg and preS1His$_6$HBcAg. His-β-L-HBcAg (Yap *et al.* 2009) served as a mother construct that gave rise to the His$_6$HBcAg and the preS1His$_6$HBcAg.

The preS1His$_6$HBcAg was produced in *E. coli* strain M15 [pREP4] in high amount (≈25 mg l^{-1} culture). However, the protein was mostly expressed in insoluble form and did not assemble into VLPs (data not shown). The insoluble preS1His$_6$HBcAg was therefore extracted and purified using the Ni^{2+} affinity HisTrap™ column in the presence of 2·5 mol l^{-1} urea. SDS-PAGE analysis of the purified preS1His$_6$HBcAg showed a similar banding profile as in Fig. 2a, lane 12. Two prominent protein bands of about 33 kDa (full-length) and 28 kDa were co-

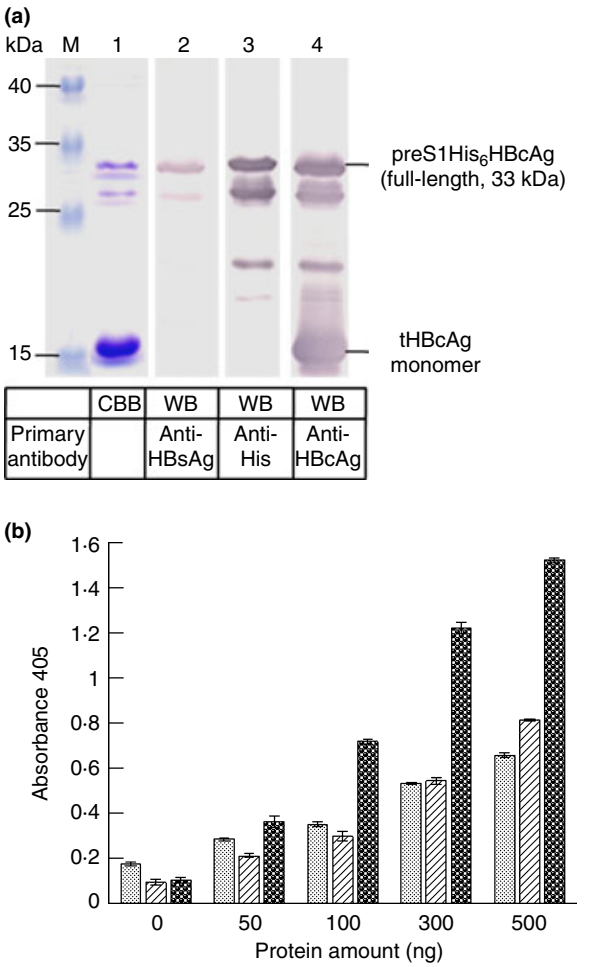

Figure 4 Antigenicity of the purified preS1His$_6$HBcAg + tHBcAg chimeric particles. The purified chimeric particles obtained from the incorporation process (tHBcAg : preS1His$_6$HBcAg ratio 4 : 1) were analysed using (a) Western blotting (WB) and (b) ELISA. Lane M, molecular weight markers (kDa); lane 1, CBB-stained gel; lane 2 and (⬚), anti-HBsAg polyclonal antibody (pAb); lane 3 and (▨), anti-His monoclonal antibody (mAb); lane 4 and (▩), rabbit anti-HBcAg serum.

purified. The 28-kDa species is the degraded product of the preS1His$_6$HBcAg during purification steps, as the band was not detected in the intact *E. coli cells* via SDS-PAGE and Western blot analysis (data not shown).

Incorporation of the preS1His$_6$HBcAg into tHBcAg particles

To display the preS1 ligand on the HBcAg VLPs, the tHBcAg subunit was employed as a helper building block to assist the coassembly of the insoluble preS1His$_6$HBcAg in the presence of 2·5 mol l^{-1} urea. The incorporation of the preS1His$_6$HBcAg in the tHBcAg particles was evaluated by mixing both different subunits in different molar ratios from 4 : 1 to 1 : 1. The level of incorporation was

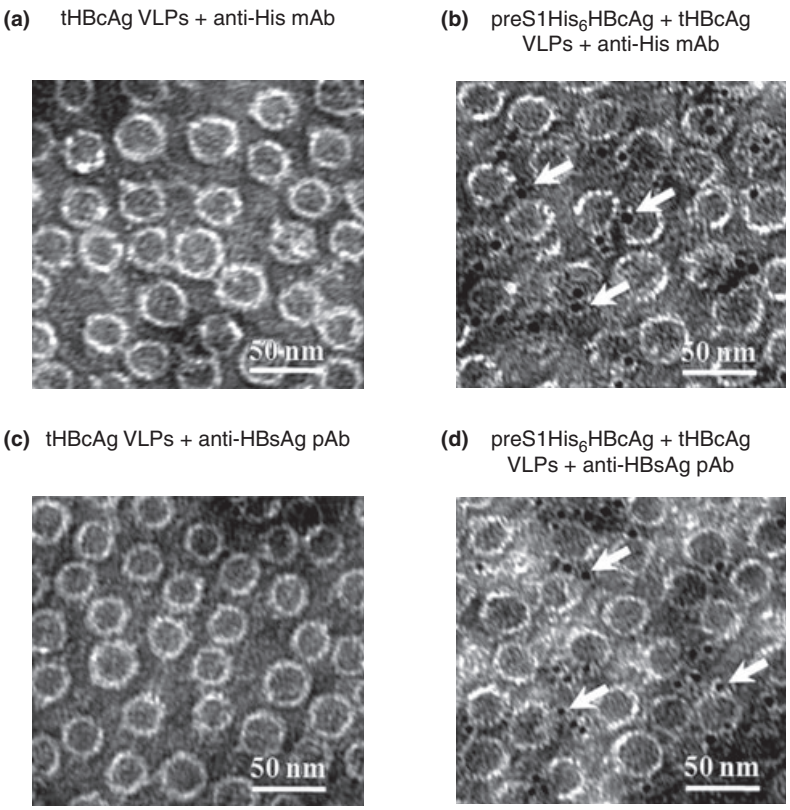

Figure 5 Colloidal gold immunoelectron micrographs of the purified preS1His$_6$HBcAg + tHBcAg chimeric particles. Electron micrographs showing the display of the hexahistidine tag and the HBV preS1$_{1\text{-}108}$ ligand on the surface of the preS1His$_6$HBcAg + tHBcAg chimeric particles. The protein samples were incubated with the anti-His mAb (b) and the anti-HBsAg pAb (d). (a) and (c) are the tHBcAg particles (negative controls) treated with the anti-His mAb and the anti-HBsAg pAb, respectively. White arrows indicate the gold particles of about 5 nm.

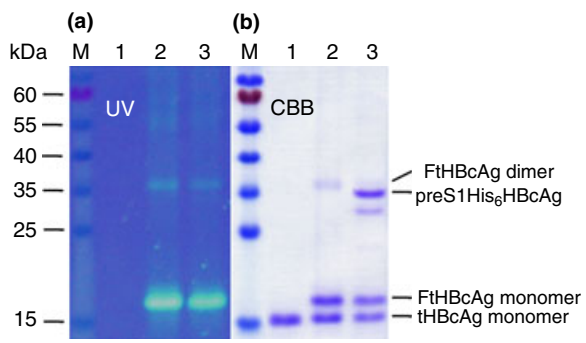

Figure 6 SDS-PAGE of the preS1His$_6$HBcAg + FtHBcAg fluorescent chimeric particles. The preS1His$_6$HBcAg subunits were incorporated into the fluorescein-labelled tHBcAg (FtHBcAg) particles in the presence of 2.5 mol l^{-1} urea. The reconstructed preS1His$_6$HBcAg + FtHBcAg fluorescent chimeric particles were purified on an 8–40% (w/v) sucrose gradient, concentrated and analysed using SDS-PAGE. Lane M, molecular weight markers (kDa); lane 1, tHBcAg control; lane 2, FtHBcAg control; and lane 3, purified preS1His$_6$HBcAg + FtHBcAg fluorescent chimeric particles. UV and CBB indicate the same gel visualized under ultraviolet illumination and CBB staining, respectively.

determined by fractionating the assembled chimeric particles, preS1His$_6$HBcAg + tHBcAg, on an 8–40% (w/v) sucrose gradient. The migration profiles of each sample were analysed using SDS-PAGE. The CBB-stained gel showed that in the absence of the helper tHBcAg monomer, the preS1His$_6$HBcAg monomer (≈33 kDa) was accumulated at the top (fractions 11–12) and did not enter the gradient (Fig. 2a). Figure 2b shows the typical migration profile of the tHBcAg particles in the gradient, forming a peak between fractions 4–9. Fractions 11 and 12 at the top of the gradient contained the unassembled tHBcAg monomer. The migration profile is in accord with that demonstrated by Tan *et al.* (2003) and Lee and Tan (2008). Interestingly, in the presence of 4 : 1 molar ratio of tHBcAg to preS1His$_6$HBcAg, an extra distinct band of about 33 kDa was found to comigrate with the tHBcAg into lower fractions (Fig. 2c). Analysis of the protein bands with an imaging system revealed that the peak was shifted slightly to a higher sucrose density (Fig. 2c) as compared with that of tHBcAg particles alone (Fig. 2b). This indicates the presence of denser particles after the

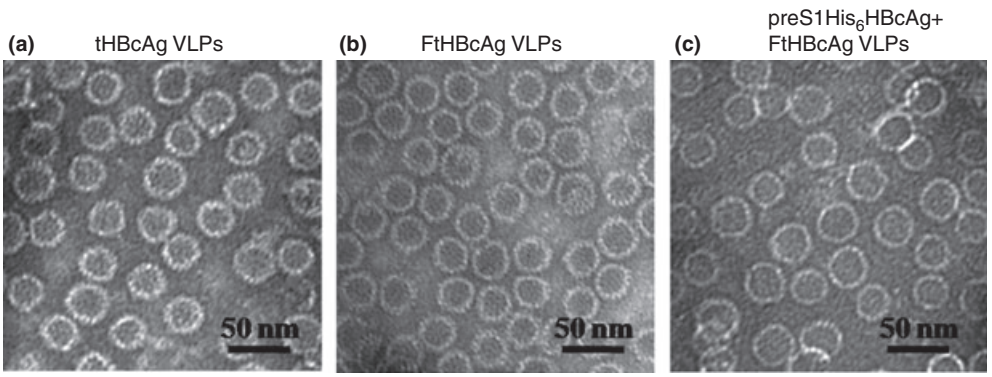

Figure 7 Electron micrographs of HBcAg particles. VLPs formed by the tHBcAg particles (a), the FtHBcAg (b) and the preS1His_6HBcAg + FtHBcAg (c). Scale bar 50 nm.

incorporation process. Similar migration profiles were also observed for the samples with 2 : 1 and 1 : 1 molar ratios of the tHBcAg to preS1His_6HBcAg (Fig. 2d,e).

To determine the level of incorporation, the fractions of the peaks (Fig. 2c–e) were pooled, concentrated and analysed by SDS-PAGE. The relative band intensity of the tHBcAg (17 kDa) and the preS1His_6HBcAg (full-length, 33 kDa) from each sample was determined. Interestingly, the reading shows that about 36% of the preS1His_6HBcAg was incorporated successfully into the tHBcAg for the sample with 4 : 1 molar ratio of tHBcAg to preS1His_6HBcAg (Fig. 3). The presence of the preS1 ligand in the preS1His_6HBcAg + tHBcAg chimeric particles was proven by Western blot analysis and ELISA (Fig. 4). The band with the molecular mass of about 33 kDa is the preS1His_6HBcAg as it was detected by the anti-HBsAg pAb, the anti-His mAb and the rabbit anti-HBcAg serum in the Western blot analysis. The bands with a smaller molecular mass (≈28 kDa) compared with that of the full-length product (≈33 kDa) could be a degraded product of the preS1His_6HBcAg as they were detected by all antibodies. The tHBcAg monomer lacking the preS1 region and the hexahistidine tag was only detected by the anti-HBcAg serum (Fig. 4a, lane 4). Besides, ELISA signals increased proportionally with the amount of coated protein (Fig. 4b), which suggests that the preS1His_6HBcAg was incorporated in the tHBcAg particles, exposing both the preS1 region and the hexahistidine tag on the surface of the chimeric VLPs. The display of the preS1 ligand on the chimeric VLPs was further proven by the colloidal gold immunoelectron microscopy using both the anti-HBsAg pAb and the anti-His mAb (Fig. 5).

Liver cell translocation assay

To evaluate the capability of the preS1 ligand in delivering fluorescein molecules into hepatocytes, the preS1His_6HBcAg was incorporated into the fluorescein-labelled tHBcAg (FtHBcAg) particles, purified and used to infect HepG2 cells. In this experiment, the preS1His_6HBcAg subunits were not labelled. Figure 6 shows the SDS-PAGE of the purified preS1His_6HBcAg + FtHBcAg fluorescent chimeric particles (Fig. 6, lane 3). The CBB-stained gel (Fig. 6b) revealed a different migration profile as compared with that of Fig. 4a, lane 1. An extra band ≈18 kDa above the tHBcAg (≈17 kDa; Fig. 6, lane 3) is the fluorescein-labelled tHBcAg (FtHBcAg). The 1-kDa shift is in good agreement with the cross-link of 2 fluorescein molecules (≈947 Da) per tHBcAg monomer. This band fluoresced under ultraviolet illumination (Fig. 6a, lanes 2 and 3). The incorporation of preS1His_6HBcAg into FtHBcAg particles and its assembly into VLP was not impaired by the fluorescein molecules in the FtHBcAg particles. TEM analysis of the FtHBcAg particles and the reconstructed preS1His_6HBcAg + FtHBcAg fluorescent chimeric particles revealed icosahedral morphology similar to the tHBcAg particles (Fig. 7). Transfection experiment demonstrated that the preS1His_6HBcAg + FtHBcAg fluorescent chimeric particles translocated into HepG2 cells and made the cell to fluoresce after 8 h of incubation at 37°C (Fig. 8b). The green fluorescent signal increased significantly when the incubation period was extended to 16 h (Fig. 8d). When the fluorescent chimeric particles were tested on HeLa cells (Fig. 8e,f), a nonhepatic cell line, no green fluorescent was observed. The translocation activity of the chimeric particles was further confirmed by detecting the presence of tHBcAg in the cytoplasm. Immunofluorescence microscopy revealed that the preS1 ligand translocated the chimeric particles into HepG2 cells instead of binding on the cell surface (Fig. 9). A strong green fluorescent signal was observed in the permeabilized cells (Fig. 9d) compared with that of the intact cells (Fig. 9c). This indicates that the preS1 ligand was exposed on the VLPs and biologically active.

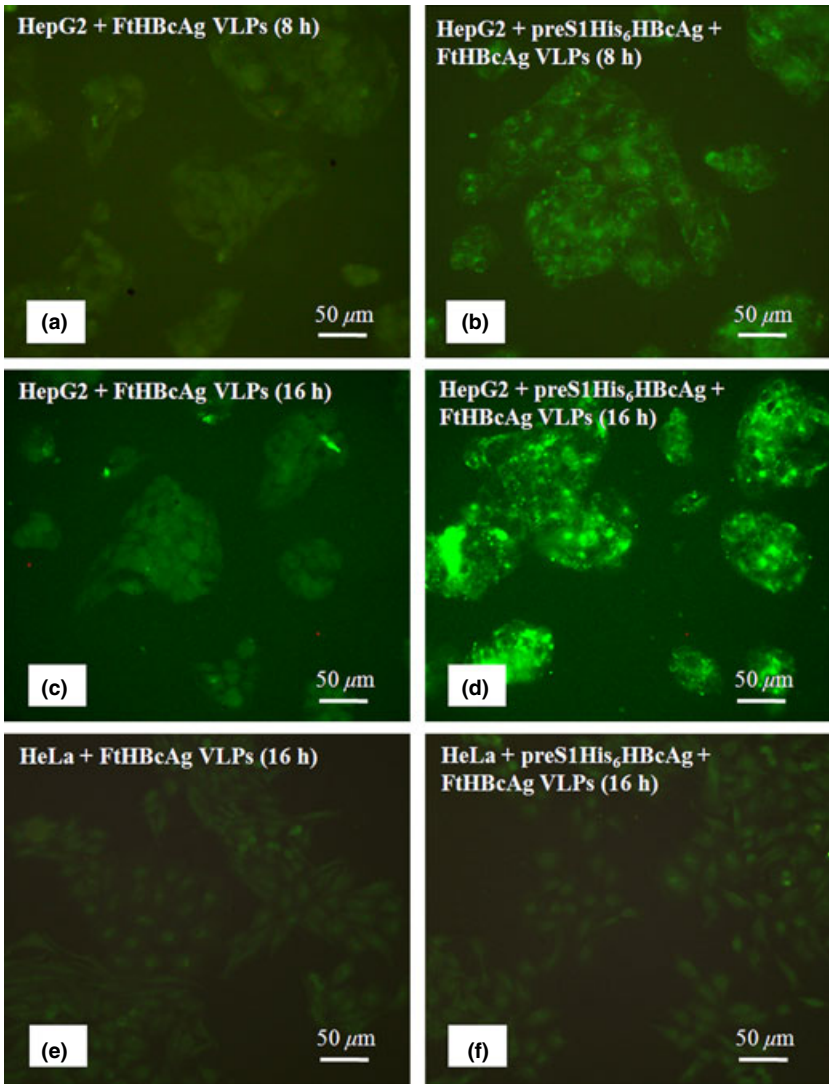

Figure 8 Translocation of the preS1His_6HBcAg + FtHBcAg fluorescent chimeric particles into HepG2 cells. (a) and (b) are HepG2 cells incubated with 25 μg of the FtHBcAg particles (negative control) and the preS1His_6HBcAg + FtHBcAg fluorescent chimeric particles, respectively, at 37°C for 8 h. (c) and (d) are HepG2 cells incubated with 25 μg of the FtHBcAg particles and the preS1His_6HBcAg + FtHBcAg fluorescent chimeric particles, respectively at 37°C for 16 h. (e) and (f) are HeLa cells (nonhepatic cells) incubated with 25 μg of the FtHBcAg particles and the preS1His_6HBcAg + FtHBcAg fluorescent chimeric particles, respectively, at 37°C for 16 h.

Discussion

HBcAg particles have been used extensively to display foreign epitopes in the development of multicomponent vaccines because of its self-assembly and antigenic properties (Whitacre *et al.* 2009; Yin *et al.* 2010). The dissociation and reassociation properties have also been studied intensively using various chemicals (Lee and Tan 2008). Recently, interest has been focused on the application of the particles as gene delivery vehicles. Cooper and Shaul (2005) demonstrated the nonspecific delivery of oligonucleotides into cells using the full-length HBcAg. A further study by the same authors (Cooper and Shaul 2006) showed that the arginine-rich region located at the C-terminal end of HBcAg is exposed partially on the VLPs and is responsible for the particles uptake through the clathrin-mediated pathway. Therefore, in the present study, the tHBcAg lacking the arginine-rich region was employed for the development of a cell delivery system.

The hepatocyte targeting system was developed by displaying the preS1 ligand on the surface of the chimeric VLPs formed by tHBcAg. The whole preS1 region was fused at the N-terminal end of the His-tagged $HBcAg_{3\text{-}148}$. The main reason was to further extend the preS1 ligand

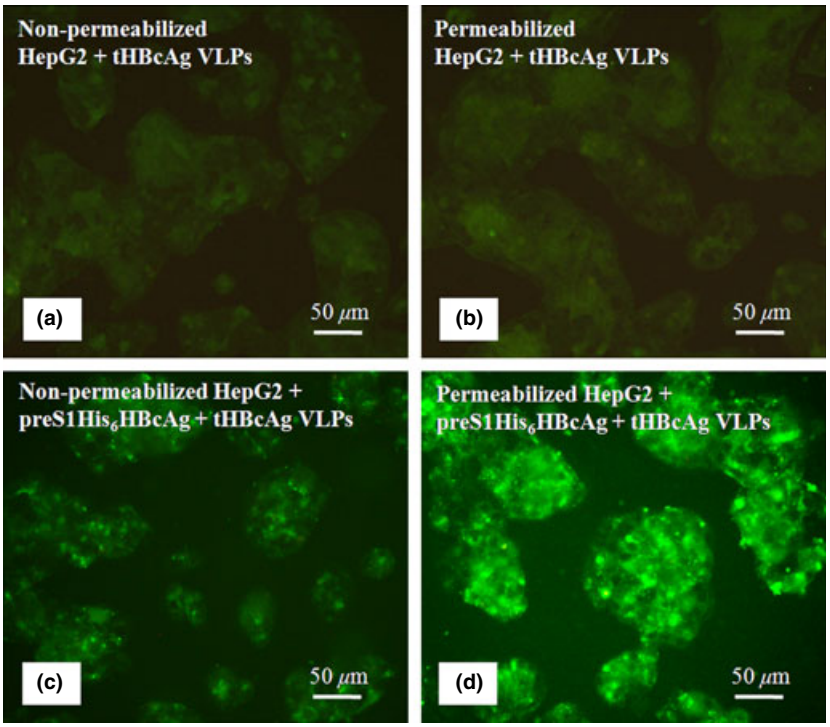

Figure 9 Detection of the internalized chimeric particles in the cytoplasm of HepG2 cells. (a) and (b) are HepG2 cells incubated with 25 μg of the tHBcAg particles (negative control). (c) and (d) are HepG2 cells incubated with 25 μg of the preS1His_6HBcAg + tHBcAg chimeric particles. After 16 h of incubation at 37°C, the cells (b, d) were permeabilized with methanol and the internalized chimeric particles were detected by rabbit anti-HBcAg serum and the DyLight™ 488 conjugated donkey anti-rabbit pAb. (a) and (c) are intact cells (nonpermeabilized) treated with the same antibodies.

exposed on the surface of the VLPs for cell receptor–ligand interaction. Recently, Yap *et al.* (2009, 2010) showed that the His-tag extended from the N-terminal region of the HBcAg was sufficient for its interaction with Ni^{2+} ligands. However, the fusion of the preS1 region to the HBcAg directly may impair particle formation because of the length of the insert and the presence of hydrophobic moieties (Kazaks *et al.* 2004). Overexpression of the preS-related proteins had produced insoluble proteins that were accumulated in inclusion bodies (Lin *et al.* 1991; Núñez *et al.* 2001). In the present study, the expression of preS1His_6HBcAg in *E. coli* also yielded insoluble proteins. To overcome this problem, several approaches have been taken to increase the solubility of the proteins: Qian *et al.* (2006) mutated the preS1 coding sequence according to *E. coli* preferred codons; Maeng *et al.* (2001) and Deng *et al.* (2005) reported the fusion of the preS1 and preS regions to glutathione S-transferase (GST), chitin-binding domain (CBD) and thioredoxin (thio) protein tags; Kazaks *et al.* (2004) coexpressed the preS region inserted in the immunodominant region of HBcAg and the tHBcAg, but the incorporated preS region was only 4–6%. Previously, the association and dissociation properties of the tHBcAg were studied (Lee and Tan 2008), and this information was used to establish a method to incorporate the insoluble preS1His_6HBcAg into the tHBcAg particles (Fig. 10a). The 4 : 1 molar ratio of tHBcAg to preS1His_6HBcAg in 2·5 mol l^{-1} urea solution dissociated the particles into their subunits. In the reassociation step, about 36% of the full-length preS1His_6HBcAg was successfully incorporated in the chimeric particles, displaying the preS1 ligands on the surface of the VLPs. In addition, the presence of 2·5 mol l^{-1} urea in the buffer maintained the stability of the purified proteins for at least 1 month at 4°C.

To date, there is still limited information available on the chemical alterations in the HBcAg VLPs. The tHBcAg possesses two lysine (K_7 and K_{96}), two aspartic acid (D_{64} and D_{78}) and one glutamic acid (E_{77}) residues for chemical alterations. Previously, HBV peptide inhibitors were cross-linked to the tips of the HBcAg VLPs using EDC and sulfo-NHS (Tang *et al.* 2007). In the current study, the amine-reactive NHS-fluorescein reagent was conjugated to the tHBcAg particles (Fig. 10b). SDS-PAGE and TEM analysis of the recombinant HBcAg particles revealed intact particles with icosahedral symmetry. When the preS1His_6HBcAg fusion protein was incorporated into

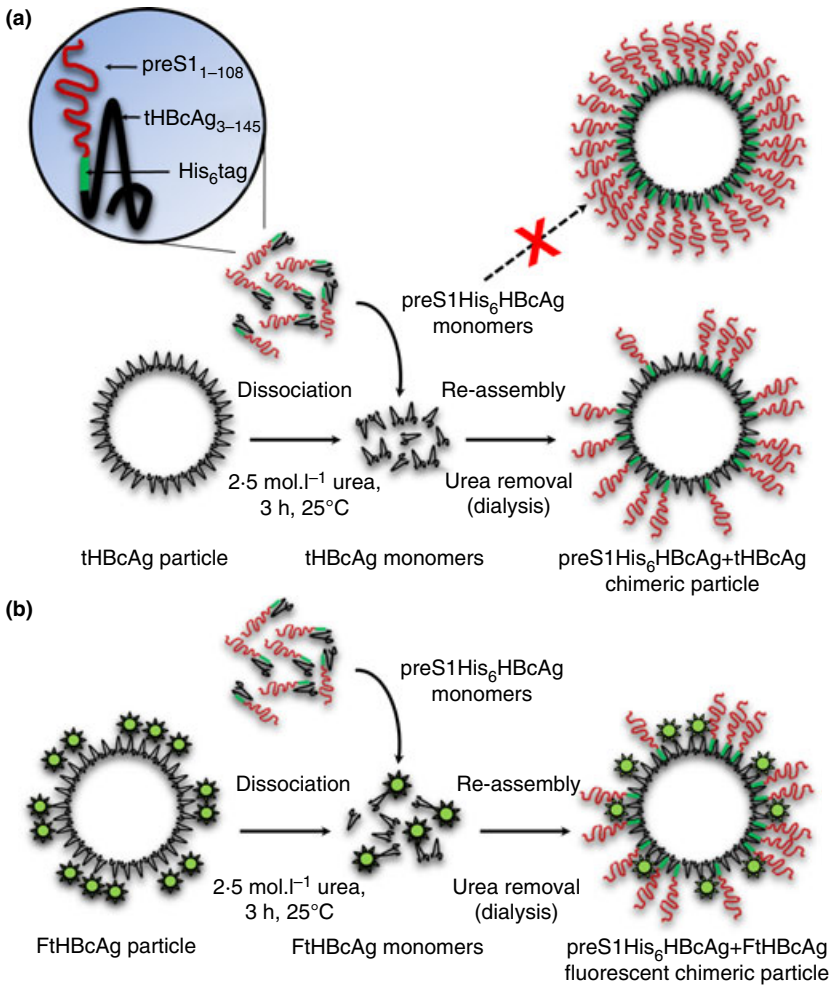

Figure 10 Schematic presentation of the production of the preS1His$_6$HBcAg + tHBcAg chimeric particles and preS1His$_6$HBcAg + FtHBcAg fluorescent chimeric particles. (a) Incorporation of the preS1His$_6$HBcAg into the truncated HBcAg (tHBcAg) particles. The preS1His$_6$HBcAg expressed in bacteria was insoluble and did not form VLPs. To induce VLP formation, purified preS1His$_6$HBcAg monomers were added to the dissociated tHBcAg subunits in the presence of 2.5 mol l^{-1} urea at 25°C. In the optimal molar ratio (4 : 1) of tHBcAg to preS1His$_6$HBcAg, about 36% of the preS1His$_6$HBcAg was incorporated successfully into the tHBcAg particles, forming icosahedral VLPs similar to those of the tHBcAg particles. The enlarged circle shows the subunit structure of the preS1His$_6$HBcAg. (b) Production of the preS1His$_6$HBcAg + FtHBcAg fluorescent chimeric particles. The purified preS1His$_6$HBcAg monomers were added to the dissociated fluorescein-labelled tHBcAg (FtHBcAg) particles as mentioned earlier. During the reassembly step, the subunits reassociate and assemble into preS1His$_6$HBcAg + FtHBcAg fluorescent chimeric particles.

the fluorescein-labelled (FtHBcAg) particles, the reconstructed preS1His$_6$HBcAg + FtHBcAg fluorescent chimeric VLPs translocated and delivered the fluorescein molecules into HepG2 cells efficiently, elucidating the functional display of the preS1 ligand on the chimeric VLPs. This could serve as a model for drug delivery into hepatocytes by substituting the fluorescein molecules with therapeutic drugs, such as doxorubicin and hygromycin (Bar *et al.* 2008; Chang *et al.* 2009). In addition, the tHBcAg can be used to package nucleic acids, antiviral agents, analogues and chemical compounds using the encapsidation method as described by Lee and Tan (2008). When the preS1His6HBcAg was incorporated into the tHBcAg VLPs containing the cargo, the preS1 ligand facilitates the delivery of the cargo to liver cells.

Myristylation of glycine residue at position 2 of the preS1 domain has been demonstrated to play an important role in HBV infectivity (Gripon *et al.* 1995; Bruss *et al.* 1996). Interestingly, De-Falco *et al.* (2001) showed that the chemically synthesized preS1 domain and its myristylated derivative interact with HBV receptor preparations derived from HepG2 cells, in which the latter displayed a 3-fold enhanced binding affinity compared with that of the preS1 alone. Recently, Tang *et al.* (2009) demonstrated that the

nonmyristylated preS1 domain displayed on bacteriophage T7 translocated HepG2 cells and delivered the phage genome into the host cells. In the present study, the nonmyristylated preS1 domain displayed on the chimeric VLPs was shown to translocate HepG2 cells.

In the present study, chimeric VLPs containing the preS1His6HBcAg and tHBcAg monomers were produced by using a newly established association and dissociation method. The liver cell–binding ligand, preS1, was proven to be displayed on the surface of the chimeric VLPs using colloidal gold immunoelectron microscopy, TEM and ELISA. Fluorescein molecules were conjugated to the chimeric VLPs and were demonstrated to transfect HepG2 cells, efficiently. The method described in this article allows the incorporation of other cell-targeting ligands into HBcAg particles that could serve as potential nanovehicles to target various cell specifically.

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