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Nanoglue: An Alternative Way To Display Cell-Internalizing Peptide at the Spikes of Hepatitis B Virus Core Nanoparticles for Cell-Targeting Delivery

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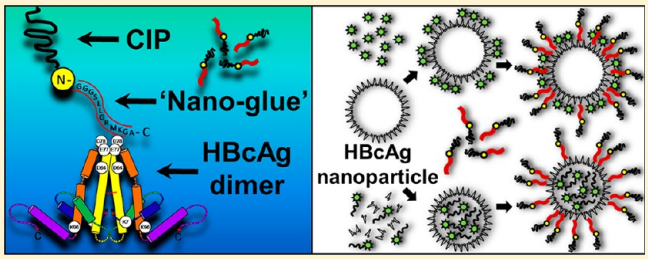
Supporting Information

ABSTRACT: Cell-internalizing peptides (CIPs) can be used to mediate specific delivery of nanoparticles across cellular membrane. The objective of this study was to develop a display technique using hepatitis B virus (HBV) capsid-binding peptide as a "nanoglue" to present CIPs on HBV nanoparticles for cell-targeting delivery. A CIP was selected from a phage display library and cross-linked specifically at the tips of the spikes of the HBV capsid nanoparticle via the "nanoglue" by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS). Fluorescent oligonucleotides packaged in the nanoparticles and the fluorescein molecules conjugated on the nanoparticles were delivered to cells by using this display technique. This study demonstrated a proof of principle for cell-targeting delivery via "nanoglue" bioconjugation.



KEYWORDS: *chemical cross-linking, conjugation, drug and gene delivery, fluorescein, hepatitis B virus, HBcAg, oligonucleotide packaging*

INTRODUCTION

Short peptides that interact specifically with cell surfaces have been employed widely for cell-targeting delivery.[1−3] With the development of phage display technology, various cell-binding peptides (CBPs) and cell-internalizing peptides (CIPs) with high affinity and specificity to hepatocarcinoma,[4−7] glioma,[8] and non-small cell lung carcinoma[9,10] were discovered. These short peptides could serve as cell-targeting ligands to be displayed on nanoparticles for drug and gene deliveries.

Hepatitis B virus (HBV) capsid which is made up of the viral core antigen (HBcAg) has been used widely as a nanocarrier for the development of multicomponent vaccines due to its self-assembly and antigenic properties.[11,12] Over three dozen foreign epitopes have been fused to HBcAg via recombinant DNA technology without impairing the capsid formation.[13,14] Apart from displaying foreign epitopes, HBV capsid has been shown to package green fluorescent protein (GFP)[15] in its large cavity of about 8,600 nm^3.[16] This lays the foundation for exploring the potential application of the capsid nanoparticle to serve as a delivery vehicle to target cells.

Biochemical and structural analyses revealed that the HBcAg subunits are clustered as dimers.[17−21] The recombinant HBcAg produced in bacteria self-assembles into large and small capsids with a diameter of about 36 and 32 nm, respectively.[22,23] The former contains 120 dimers, the latter consists of 90 dimers, and each dimer forms a spike on the surface of the capsids.[20,21,23] HBcAg comprises 183 amino acids, and its C-terminal end is highly rich in arginine residues.[24,25] Recently, this region has been reported to drive the cellular uptake of the capsids in a cell-type independent manner via clathrin-mediated endocytosis.[26,27] A HBcAg mutant, lacking the C-terminal arginine rich region, namely, truncated HBcAg (tHBcAg), is

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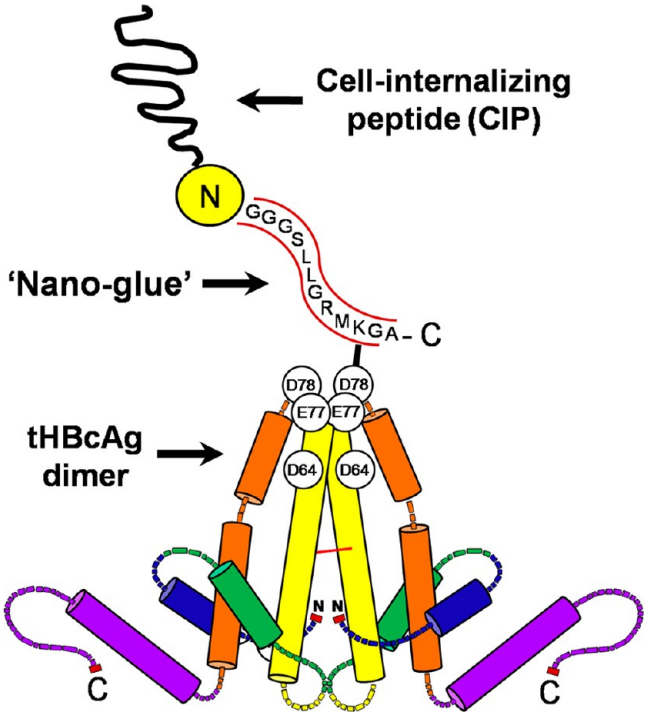
able to self-assemble into capsid,[20,21,28] but it cannot translocate cell membrane.[27] Hence, a specific cell-targeting ligand displayed on the surface of tHBcAg nanoparticle is strongly believed to transform the nanoparticles into a cell delivery system. Polypeptides to be displayed on HBV capsid can be fused either at the N-terminal end or in the immunodominant region (residues 78−82) of the HBcAg via DNA cloning.[29−34] However, this approach is complicated, labor intensive, and time-consuming. In the present study, we have established a novel method to display CIPs on the tHBcAg nanoparticles via a HBV capsid-binding peptide or known as the "nanoglue". A decapeptide (GSLLGRMKGA) which interacts specifically at the tips of the spikes of the HBcAg nanoparticles was employed as a "nanoglue" to display CIPs (Scheme 1).

Scheme 1. Schematic Representation of the Nanoglue Concept[a]



[a]Interaction of the nanoglue at the tip of a tHBcAg dimer, displaying the CIPs on the surface of the tHBcAg nanoparticles. In the presence of EDC and sulfo-NHS, amino group from lysine residue of the nanoglue cross-links the adjacent carboxyl group from aspartic acid (Asp-64, Asp-78) or glutamic acid (Glu-77) residues located at the tip of the dimer.

The decapeptide (GSLLGRMKGA) was originated from the LLGRMK peptide isolated from biopanning a phage display peptide library against HBV capsid.[35,36] Both the peptides bind at the tips of HBcAg and tHBcAg. In the presence of EDC and sulfo-NHS, the primary amine group from the lysine residue of the decapeptide cross-links the adjacent primary carboxyl group from Asp-64, Glu-77, or Asp-78 of HBcAg to form an amide bond.[36,37] Therefore, a CIP fused to the N-terminal end of the decapeptide is hypothesized to bind at the tip and display on the surface of the HBcAg nanoparticles for cell delivery.

EXPERIMENTAL SECTION

Selection of Cell-Internalizing Peptides (CIPs). CIPs were selected by biopanning the Ph.D.-12 Phage Display Peptide Library (NEW ENGLAND BioLabs Inc., Ipswich, MA, USA) against HeLa cells. The peptide library is based on a combinatorial library of random dodecapeptides fused to the minor coat protein (gp3) of M13 filamentous phage. HeLa cells (1,200,000 cells) were seeded onto a tissue culture dish (10 cm) in 5 mL of DMEM medium (Sigma, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma, St. Louis, MO, USA). The cells were incubated at 37 °C for 16 h in a humidified atmosphere containing 5% CO_2. After incubation, the medium was changed and a total of 1.5×10^{11} virions from the peptide library was added to the cells and incubated for 5 h. The medium was later removed and the infected cells were washed 6 times with 10 mL of phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4; pH 7.4). The cells were harvested, resuspended in 5 mL PBS (pH 7.4) containing 0.1 mg/mL proteinase K, and incubated at 4 °C for 1 h to inactivate the surface bound phages. The cells were washed another 2 times with 10 mL of ice cold PBS (pH 7.4) and resuspended in 500 μL of TBS buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl] containing 10 mM phenylmethylsulfonyl fluoride (PMSF). The cells were then lysed by vortexing after the addition of lysis buffer [2% (w/v) sodium deoxycholate, 10 mM Tris-HCl (pH 8.0), 2 mM ethylenediaminetetraacetic acid (EDTA); 500 μL]. The infectious phages in the cell lysate were then amplified and used for the subsequent two rounds of biopanning as mentioned above. The infectious phages were determined by plating out an aliquot of the cell lysate recovered from each round of biopanning using *Escherichia coli* strain ER2738. The nucleotide sequence of the isolated clones was determined by sequencing the gp3 gene carrying the insert.[38]

Immunofluorescence Microscopy. In order to study the internalization property of the isolated M13 clones, the phages were amplified and tested on HeLa and HepG2 (negative control) cells. The cells were seeded (200,000 cells/well) onto sterile glass coverslips in a six-well plate (TPP, Trasadingen, Switzerland) and grown in DMEM medium (Sigma, St. Louis, MO, USA) containing 10% (v/v) fetal bovine serum (FBS; Sigma, St. Louis, MO, USA) at 37 °C. After 16 h of incubation, the medium was changed and the purified phages (1.5×10^{12} virions) were added to the cells accordingly. The cells were incubated at 37 °C for another 72 h. After that, 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 mouse anti-M13 monoclonal antibody (1:100 dilutions in PBS containing 0.2 mg/mL BSA, 1 h, 25 °C; GE Healthcare Life Sciences, Buckinghamshire, U.K.), followed by the FITC conjugated goat anti-mouse antibody (1:100 dilutions in PBS containing 0.2 mg/mL BSA, 1 h, 25 °C; Amersham Biosciences, Solon, USA) in the dark. The cells were then mounted onto a drop of mounting medium [90% (v/v) glycerol, 20 mM Tris-HCl (pH 8.5), 0.1 M propyl gallate] on a glass slide, sealed with nail polish, and observed under a fluorescence microscope. In this experiment, the untreated cells and the cells added with the M13KE wild-type phage were used as negative controls.

Peptide Synthesis. The KLHISKDHIYPTGGGSLL-GRMKGA (JG24) peptide was synthesized based on the 9-

Table 1. HeLa Cell-Internalizing Peptides Obtained from Biopanning

	round 1		round 2		round 3	
dodecapeptide sequences	freq	percentage (%)	freq	percentage (%)	freq	percentage (%)
KLHISKDHIYPT	0	0.0	1	8.3	10	41.7
HTLQIPQHATSF	0	0.0	3	25.0	8	33.3
THASKNTSYFV	0	0.0	1	8.3	4	16.7
unrelated sequences	12	100.0	7	58.3	2	8.3

fluorenylmethoxycarbonyl (Fmoc) solid-phase chemistry, using the Apogee automated peptide synthesizer (AAPPTec, Louisville, KY, USA). The peptide sequence was assembled on a Rink amide resin (0.5 g, 0.7 mmol/g loading capacity; AAPPTec, Louisville, KY, USA). The coupling process and the Fmoc deprotection cycles were carried out in *N*,*N*-dimethylformamide (DMF), using HCTU/DIPEA as an activator and 20% (v/v) piperidine as a deprotecting agent accordingly. After the synthesis, the peptide was cleaved from the resin using a cleavage cocktail [90% (v/v) trifluoroacetic acid, 1% (v/v) triisopropyl silane, 5% (v/v) phenol, 2.5% (w/v) dithiothreitol; 7.5 mL for 2 h at 25 °C]. The cleaved peptide was separated from the resin and precipitated with cold ether (10 volumes) to yield the crude peptide. After precipitation, the peptide pellet was dissolved in 20 mL of ultrapure water and extracted 3 times with chloroform (equal volume) to remove trace amount of hydrophobic impurities. The partially purified peptide was lyophilized, and the purity of the peptide was checked with an analytical HPLC using a C-18 column. The molecular mass of the peptide was determined by SYNAPT G2 high definition mass spectrometry (Waters, Milford, MA, USA) and found to be 2535.1 Da.

Chemical Cross-Linking of KLHISKDHIYPTGGGSLLGRMKGA Peptide to tHBcAg Nanoparticles. The cross-linking of the KLHISKDHIYPTGGGSLLGRMKGA (JG24) peptide at the spike of tHBcAg nanoparticles was achieved by incubating different molar ratio of tHBcAg to JG24 (8:1, 4:1, 2:1, 1:1, 1:2, 1:4, 1:8, and 1:10) in the presence of chemical modification reagents [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, EDC; and *N*-hydroxysulfosuccinimide, sulfo-NHS] as described by Böttcher et al.[36] and Tang et al.,[37] with some modifications. tHBcAg (60 μg) was incubated with the JG24 peptide for 16 h at 4 °C in 60 μL of reaction buffer (25 mM sodium phosphate; pH 7.0, 150 mM NaCl, 1.8 mM EDC, 1.8 mM sulfo-NHS). Later, the optimum incubation time needed for the reaction to reach its saturation point was obtained by selecting a suitable tHBcAg:JG24 ratio for a time course study. The percentage of the tHBcAg which cross-linked successfully with the JG24 peptide (tHBcAg-JG24) was determined by measuring the relative intensity of the protein bands for both the tHBcAg (≈17 kDa) and the tHBcAg-JG24 (≈20 kDa) on a coomassie brilliant blue (CBB) stained gel, using the GelDoc 2000 imaging system (Bio-Rad, Philadelphia, PA, USA).[39]

Transmission Electron Microscopy (TEM). In the preparation for TEM analysis, protein samples (≈0.3 mg/mL; 15 μL) were absorbed onto carbon coated grids (200 mesh) and stained with uranyl acetate [2% (w/v)].[15] The grids were observed under a TEM (Philips HMG 400).

Fluorescein Labeling of the tHBcAg Nanoparticles. The tHBcAg was labeled at the primary amine groups of lysine side chain at positions 7 (Lys-7) and 96 (Lys-96) using the amine-selective NHS-fluorescein reagent (Pierce, Rockford, IL, USA). The labeling procedure was performed as recommended by the manufacturer with some modifications. The NHS-fluorescein (15 molar in excess) was dissolved in dimethylsulfoxide (DMSO) and incubated with the purified tHBcAg (5 mg/mL) in PBS (pH 7.4) for 16 h at 4 °C. The nonreacted NHS-fluorescein molecules were removed by dialysis against PBS (pH 7.4) at 4 °C. In this experiment, the labeling reaction was performed at pH 7.4 to avoid the protonation of the α-amine at the N-terminal end of tHBcAg. This limits the labeling of fluorescein molecules at the ϵ-amine of the lysine side.[40]

Delivery of Fluorescein Labeled tHBcAg (FtHBcAg) Nanoparticles into HeLa Cells. The cross-linking of JG24 peptide to FtHBcAg was performed by adding FtHBcAg:JG24 (1:2 ratio) to the reaction buffer and incubated at 4 °C for 6 h. The conjugation of the peptide to FtHBcAg was confirmed by SDS−PAGE, and its icosahedral structure was observed under TEM. In order to evaluate the internalization activity of the JG24 peptide displayed on the FtHBcAg nanoparticles in delivering fluorescein molecules into cells, the FtHBcAg-JG24 sample was dialyzed against PBS (pH 7.4) to remove the excessive cross-linkers, concentrated, and applied to HeLa cells *in vitro*. The cells were incubated at 37 °C for an indicated time, fixed with paraformaldehyde, and observed under a fluorescence microscope at appropriate magnification. In this experiment, both the FtHBcAg nanoparticles and FtHBcAg nanoparticles reacted with cross-linkers were used as negative controls. To rule out staining artifacts due to paraformaldehyde fixation, this experiment was repeated by omitting the fixation step (see Supporting Information for further details).

Delivery of Fluorescein Labeled Oligonucleotides by tHBcAg Particles. The oligonucleotides labeled with two fluorescein molecules, each at the 5′-end and the 3′-end, were produced by Bio Basic Inc. (5′ FAM-AAGCTTTCCCCAGCATGCCGGCATGCTGGGGAAA-FAM 3′; 34 bases; Ontario, Canada). The encapsidation of the fluorescent oligonucleotides by the tHBcAg nanoparticles was achieved by the dissociation and reassociation technique.[15] tHBcAg (5 mg, 20 mL) was incubated at 25 °C for 3 h in the presence of 2.5 M urea to dissociate the particles. After that, fluorescent oligonucleotides were added to the dissociated tHBcAg solution (molar ratio tHBcAg:oligonucleotide, 2:1). The reassociation of the nanoparticles was performed by removing the urea via dialysis. The tHBcAg particles harboring the fluorescent oligonucleotides were purified on sucrose density gradient ultracentrifugation [8−40% (w/v), 210000*g*, 4 °C, 5 h]. The nanoparticles were then cross-linked with the JG24 peptide as mentioned above and applied to HeLa cells *in vitro*. In this experiment, the free fluorescent oligonucleotides and the tHBcAg nanoparticles (without JG24 peptide) packaged with fluorescent oligonucleotides were used as negative controls.

RESULTS

Selection of HeLa Cell-Internalizing Peptide. HeLa CIPs were selected from a phage display random dodecapeptide library via biopanning. In the selection process, serine

endopeptidase (proteinase K) was used to partially digest the gene 3 protein (gp3) of the M13 phage, eliminating the cell surface bound phages from the library. The infectious phage clones that internalized and survived in the cells were released by cell lysis. The DNA inserts isolated from first (12 individual phage clones), second (12 individual phage clones), and third (24 individual phage clones) rounds of biopanning were sequenced, and the deduced peptide sequences are shown in Table 1.

Three dominant peptide sequences, KLHISKDHIYPT, HTLQIPQHATSF, and THASKNTSYFV, were isolated from the library. The cell internalization activity of each of the isolated phage clones was confirmed by infecting the HeLa cells with the purified phages. Immunofluorescence microscopy showed that all the phages carrying the KLHISKDHIYPT (Figure 1a), HTLQIPQHATSF (Figure 1b), and THASKNTSYFV (Figure 1c) internalized HeLa cells after 72 h of incubation at 37 °C. Interestingly, when these phages were tested on HepG2 cells, no fluorescent signals were observed (Figure 1). This shows that the isolated phage clones internalized HeLa cells, but not HepG2 cells.

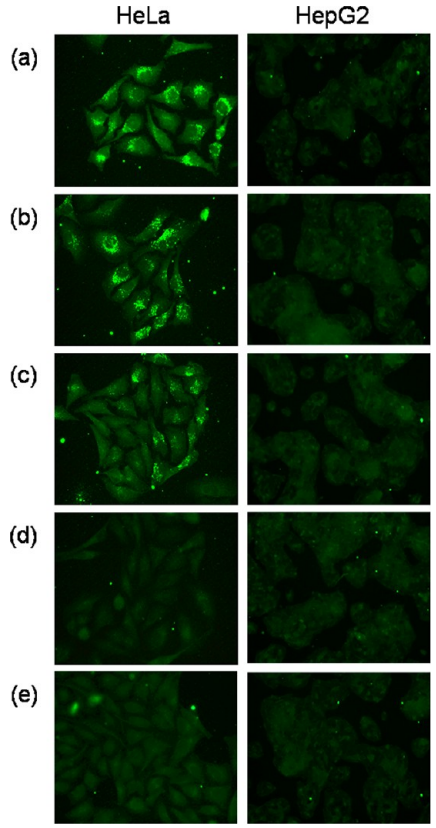


Figure 1. Immunofluorescence microscopic analysis of cell internalization activity of phage clones. Purified phage (1.5×10^{12} virions) carrying the dodecapeptides, (a) KLHISKDHIYPT, (b) HTLQIPQHATSF, (c) THASKNTSYFV, and (d) M13KE wild-type M13 phage (negative control), was added to HeLa and HepG2 cells. (e) No phage was added (negative control). After 72 h of incubation at 37 °C, the cells were treated with mouse anti-M13 monoclonal antibody, followed by FITC conjugated goat anti-mouse antibody.

Cell-Internalization Activity of the Free Peptide. In order to prove the "nanoglue" concept to display CIPs on the tHBcAg nanoparticles, the most dominant peptide sequence, KLHISKDHIYPT, obtained from the biopanning was chosen as a model peptide for the entire study. First, the internalization activity of the free peptide in the absence of phage particle was examined. KLHISKDHIYPT peptide was synthesized chemically and labeled with a fluorescein at its N-terminal end. The free peptide was then added to HeLa cells, and the internalization of the peptide into the cells was examined by fluorescence microscopy. In this experiment, the GGGSLLGRMKGA peptide which binds specifically to the tHBcAg nanoparticles was used as a negative control. In the absence of the gp3 protein, KLHISKDHIYPT peptide was demonstrated to internalize HeLa cells *in vitro* (Figure 2b). No internalization was observed for the control peptide, GGGSLLGRMKGA (Figure 2a).

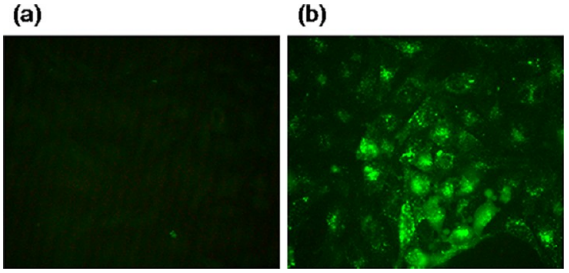


Figure 2. Internalization of synthetic peptides into HeLa cells. HeLa cells were incubated with fluorescein labeled (a) GGGSLLGRMKGA (negative control) and (b) KLHISKDHIYPT at 37 °C for 16 h.

Chemical Cross-Linking of CIP via Nanoglue. The biologically active KLHISKDHIYPT peptide was then cosynthesized with the nanoglue sequence at its C-terminal end (KLHISKDHIYPTGGGSLLGRMKGA; JG24) and cross-linked at the tips of the tHBcAg nanoparticles. The underlined glycine residues were added between the peptides to increase the flexibility of the displayed CIP. Figure 3a shows that the cross-linking activity increased from about 15% to 29% when the molar ratio of tHBcAg to JG24 was increased from 8:1 to 1:2 after being incubated at 4 °C for 16 h. The increment of the JG24 peptide molar ratio (tHBcAg:JG24) to 1:4, 1:8, and 1:10 did not show any significant enhancement in the cross-linking level. When the sample with tHBcAg to JG24 molar ratio of 1:2 was analyzed with different incubation time points, the cross-linking reaction reached its saturation point (≈31%) after 6 h of incubation at 4 °C (Figure 3b). This is in good agreement with the cross-linking of the peptide, MHRSLLGRMKGA (K_d^{rel} = 0.55 ± 0.03 nM), to tHBcAg in which the reaction yielded less than 50% of the end product at the optimum condition. Only one peptide was cross-linked to a dimer of HBcAg located close to the 2-fold axis of the nanoparticle.[36] A band shift corresponding to about 2.5 kDa on the SDS−polyacrylamide gel (Figure 4a, lane 3) indicates the cross-linking of the JG24 peptide to the tHBcAg monomers. Electron microscopic analysis revealed that the cross-linking of the JG24 peptides at the spikes had no adverse impact upon the physical properties of the tHBcAg nanoparticles (Figure 4d). However, when the above optimized condition was used to cross-link the KLHISKDHIYPT peptide alone, no cross-linking activities were observed, as the SDS−polyacrylamide gel did not show any band shift profile (see Supporting Information for further details).

Conjugation of Fluorescein and JG24 Peptide on tHBcAg Nanoparticles. The tHBcAg contains two lysine residues at positions 7 (Lys-7) and 96 (Lys-96) which can be

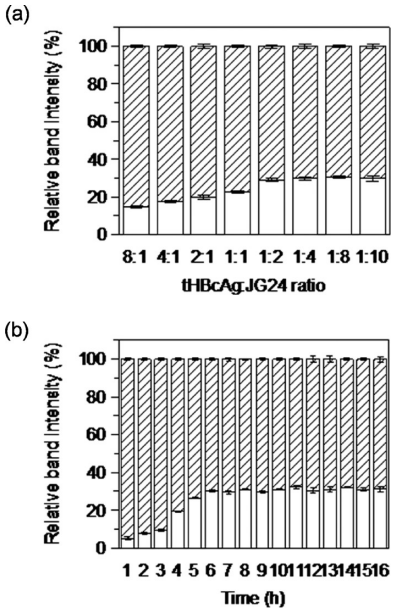


Figure 3. Chemical cross-linking of the JG24 peptide to the tHBcAg nanoparticles. Relative band intensity of tHBcAg (hatch lines) and tHBcAg cross-linked with the JG24 peptide (JG24-tHBcAg; no hatch lines) from a coomassie brilliant blue (CBB) stained SDS–polyacrylamide gel was determined by using the GelDoc 2000 imaging system. (a) Cross-linking profile of the tHBcAg nanoparticles (60 μg) with the JG24 peptide in different molar ratios of tHBcAg to JG24 (from 8:1 to 1:10) at 4 °C for 16 h. The percentage of JG24-tHBcAg increased from 14.7 ± 0.7% to 29.1 ± 0.8% for samples with the tHBcAg:JG24 molar ratio of 8:1 and 1:2, respectively. Samples with a higher JG24 molar ratio (tHBcAg:JG24, 1:4–1:10) showed no significant enhancement in the cross-linking (30.1 ± 0.4%). (b) Cross-linking profile of the sample with a tHBcAg:JG24 molar ratio, 1:2 at 4 °C. The cross-linking activity increased from 5.1 ± 0.7% after 1 h incubation and reached its saturation point (30.9 ± 0.9%) after 6 h of incubation. Data points are mean ± standard deviation of triplicate determinations.

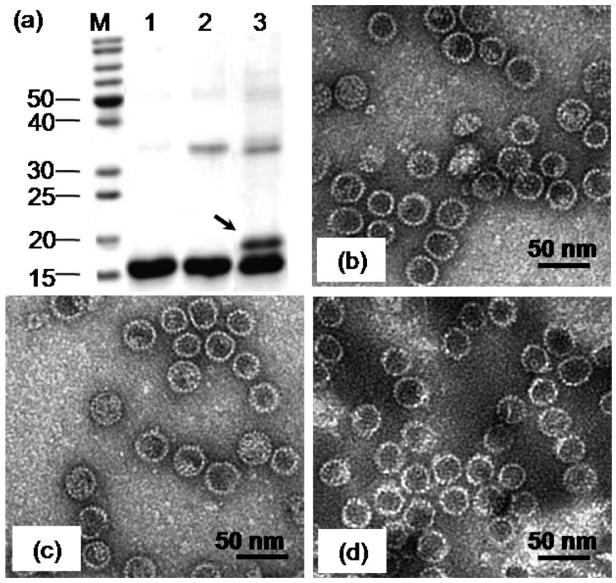


Figure 4. SDS–polyacrylamide gel and electron micrographs of the tHBcAg nanoparticles cross-linked with double molar excess of the JG24 peptide at 4 °C for 6 h. (a) SDS–polyacrylamide gel stained with CBB. Lane M, molecular mass markers (kDa); lane 1, tHBcAg control; lane 2, tHBcAg plus cross-linkers; and lane 3, tHBcAg plus the JG24 peptide and cross-linkers. Arrow indicates a shifted band equivalent to about 2.5 kDa above the 17 kDa tHBcAg. (b), (c), and (d) are electron micrographs of the samples from lanes 1, 2, and 3, respectively.

conjugated with amine selective reagents. It is therefore of interest to evaluate the functionality of these bioconjugation sites to carry therapeutic molecules for drug delivery. In this experiment, the NHS-fluorescein was chosen as a model cargo in order to make the tHBcAg nanoparticles fluorescent. The tHBcAg was conjugated with the NHS-fluorescein at Lys-7 and Lys-96 (FtHBcAg) and cross-linked with the JG24 peptide using EDC and sulfo-NHS. SDS–PAGE analysis showed that about 50% of the tHBcAg was labeled with fluorescein molecules. An extra band, about 18 kDa, above the tHBcAg (≈17 kDa) in Figure 5a, lane 1, is the fluorescein labeled tHBcAg (FtHBcAg). The 1 kDa shift is in good agreement with the conjugation of two fluorescein molecules (≈947 Da) per tHBcAg subunit. When the JG24 peptides were cross-linked to FtHBcAg nanoparticles, denaturing SDS–polyacrylamide gel shows that the peptides were cross-linked to tHBcAg subunits conjugated with either one or two fluorescein molecules (Figure 5a, lane 3). The CBB-stained SDS–polyacrylamide gel revealed a different banding profile as observed in Figure 4a, lane 3. Another two extra bands, about 20.5 kDa and 21 kDa, are the FtHBcAg cross-linked with the JG24 peptide (≈2.5 kDa), each with the tHBcAg conjugated with one and two fluorescein molecules, respectively. These bands have fluoresced under ultraviolet illumination (Figure 5a, lane 3; UV). Surprisingly, conjugation with fluorescein molecules at Lys-7 and Lys-96 did not affect the nanoparticle morphology (Figure 5d).

Delivery of Fluorescent tHBcAg (FtHBcAg) Nanoparticles into HeLa Cells. When these fluorescent nanoparticles displaying the CIPs were tested on HeLa cells, fluorescence microscopy showed that the CIPs were biologically active and the nanoparticles were internalized into the cells after 16 h of incubation at 37 °C by displaying strong green fluorescent dots (Figure 6c).

Packaging and Delivery of Fluorescent Oligonucleotides into Cells. In order to evaluate the tHBcAg nanoparticles as a cargo delivery system, the nanoparticles were packaged with fluorescent oligonucleotides and targeted to HeLa cells. An UV illuminated native agarose gel showed that the tHBcAg nanoparticles packaged with fluorescent oligonucleotides were migrated and separated into two distinct fluorescent bands, which correspond to the $T = 3$ and $T = 4$ tHBcAg nanoparticles (Figure 7). The tHBcAg nanoparticles packaged with fluorescent oligonucleotides have fluoresced under UV illumination (Figure 7, lane 2). The migration profile is in good agreement with that demonstrated previously for the packaging of GFP by the tHBcAg nanoparticles.[15] When the tHBcAg nanoparticles harboring the fluorescent oligonucleotides were cross-linked with the JG24 peptides and added to HeLa cells, the cells fluoresced under the fluorescence microscope (Figure 8c). This shows that the cargo, fluorescent oligonucleotides, was delivered successfully into the cells by tHBcAg nanoparticles displaying the CIP.

■ DISCUSSION

A peptide that interacts specifically with a virus capsid is hypothesized to serve as a ligand or "glue" to display a mammalian cell binding peptide on the surface of the virus

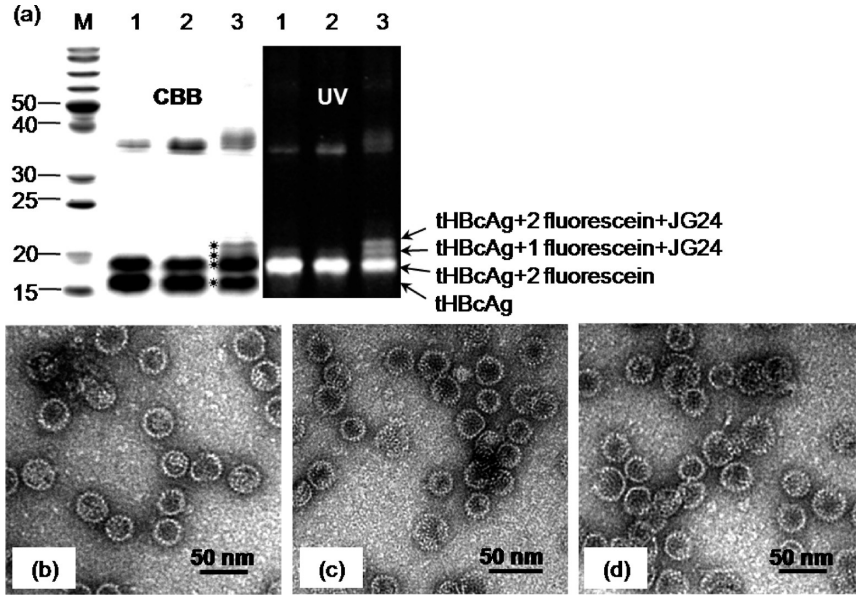


Figure 5. Chemical cross-linking of the JG24 peptide to fluorescein labeled tHBcAg (FtHBcAg) nanoparticles. (a) SDS−polyacrylamide gel. Lane M, molecular weight markers (kDa); lane 1, FtHBcAg control; lane 2, FtHBcAg plus cross-linkers; and lane 3, FtHBcAg plus the JG24 peptide and cross-linkers. CBB and UV indicate the same gel visualized under coomassie brilliant blue staining and ultraviolet illumination, respectively. (b), (c), and (d) are electron micrographs of the samples from lanes 1, 2, and 3, respectively.

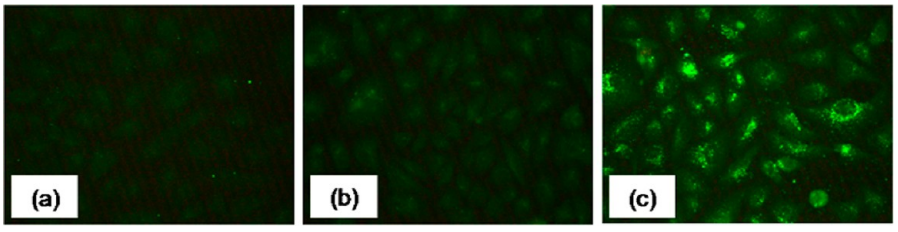


Figure 6. Delivery of fluorescein molecules into HeLa cells. Samples from Figure 5 were prepared, dialyzed (to remove excess cross-linkers), and added to HeLa cells accordingly (150 μg). The cells were incubated at 37 °C for 16 h. (a) FtHBcAg nanoparticles, (b) FtHBcAg nanoparticles plus cross-linkers, and (c) FtHBcAg naoparticles plus the JG24 peptide and cross-linkers.

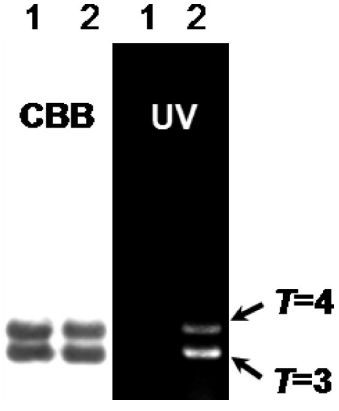


Figure 7. Native agarose gel [1.5% (w/v)] electrophoresis of the tHBcAg nanoparticles packaged with fluorescent oligonucleotides. Lane 1, tHBcAg control; lane 2, tHBcAg packaged with fluorescent oligonucleotides. CBB and UV indicate the same gel visualized under coomassie brilliant blue staining and ultraviolet illumination, respectively.

nanoparticle for cell targeting delivery. In order to prove this hypothesis, HBV capsid was chosen as a model in this study because it can be produced easily in *E. coli* and its three-dimensional structure has been determined by electron cryomicroscopy[23] and X-ray crystallography.[20,21]

A ligand with the sequence LLGRMK was isolated from a phage display library via biopanning against tHBcAg capsid.[35] This peptide interacts specifically with tHBcAg and inhibits HBV viral assembly by blocking the interaction between the viral capsid and surface proteins.[35,36] A derivative of this peptide bearing the sequence GSLLGRMKGA and with an enhanced binding affinity was demonstrated to bind at the tips of the capsid spikes by using electron cryomicroscopy.[36] The lysine residue of the peptides interacts with the aspartic acid or glutamic acid residue located at the tips of HBcAg nanoparticles to form an amide bond in the presence of cross-linker reagents (EDC and sulfo-NHS).[36,37] These reagents link the adjacent primary amino group from the lysine residue and primary carboxyl group from the aspartic acid or glutamic acid residues. Therefore, the peptide and reagents permit a cell-targeting ligand to be cross-linked to the tHBcAg nanoparticles (Scheme 1).

In the current study, HeLa CIPs were selected from a phage display library. These CIPs are different from the trans-activating transcriptional activator (TAT) of HIV-1. TAT contains a protein transduction domain (PTD) with the sequence YGRKKRRQRRR which allows the TAT to internalize a cell membrane.[41] The nuclear localization signal, GRKKR,

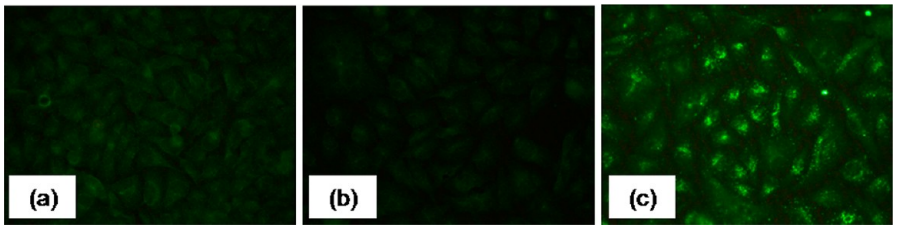
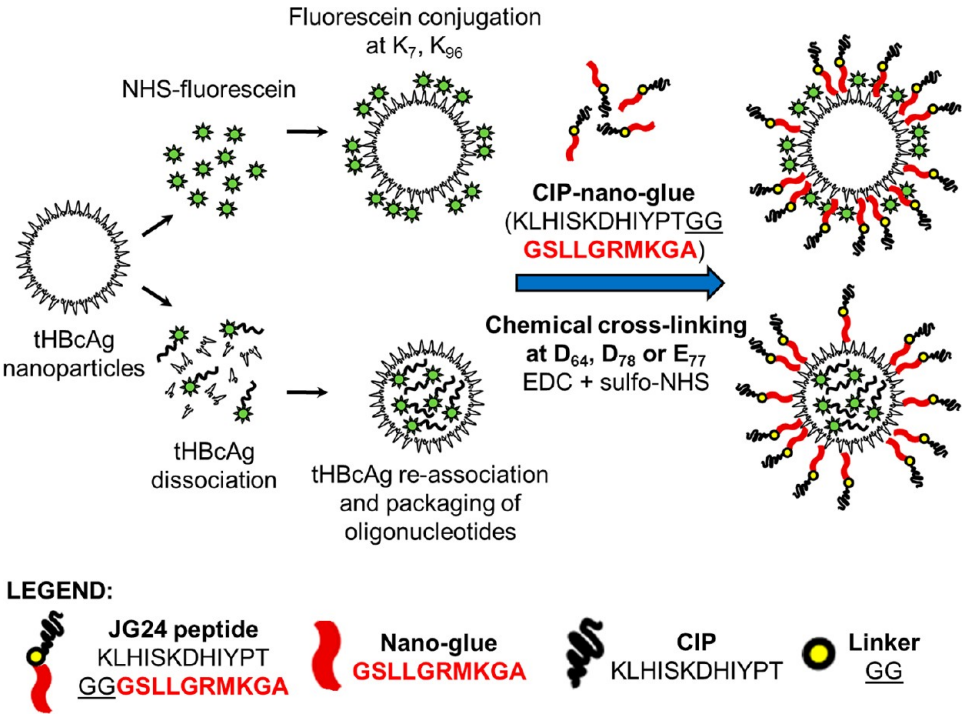


Figure 8. Delivery of fluorescent oligonucleotides into HeLa cells. tHBcAg nanoparticles were cross-linked with the JG24 peptide using EDC and sulfo-NHS. HeLa cells were incubated with 4 μM of free oligonucleotides (a), tHBcAg nanoparticles (150 μg) packaged with fluorescent oligonucleotides (b), and tHBcAg nanoparticles (150 μg) packaged with fluorescent oligonucleotides and cross-linked with the JG24 peptide (c).

Scheme 2. Schematic Representation of the tHBcAg Nanoparticles as a Delivery Transport Using the Nanoglue Display Technique[a]



[a]The tHBcAg possesses two lysine residues (Lys-7 and Lys-96) for conjugation of molecules using an amine selective reagent. During the dissociation and reassociation of the tHBcAg nanoparticles, cargo can be packaged in the inner cavity of the nanoparticles. By using the nanoglue technique, the tHBcAg nanoparticles displaying the CIPs are biologically active and are able to internalize cells.

found within the PTD, mediates further translocation of TAT into the cell nucleus.[42] The PTD internalizes mammalian cells nonspecifically, including animal tissues and the brain.[43] On the other hand, the CIP with the sequence KLHISKDHIYPT was found to internalize HeLa cells and was detected in the endoplasmic reticulum (ER) of the cells (see Supporting Information for further details).

In order to elucidate the nanoglue concept, the most dominant peptide, KLHISKDHIYPT, was used as a cell-targeting ligand to be displayed on the tHBcAg nanoparticles by using the nanoglue concept. The CIP, KLHISKDHIYPT, either coupled with the M13 phage or existing alone as a free peptide showed cell-internalizing activity to internalize HeLa cells. Therefore, the KLHISKDHIYPT peptide sequence was ligated with the nanoglue (GGGSLLGRMKGA) at the C-terminus and conjugated covalently at the tips of the tHBcAg nanoparticles (Scheme 1). Two glycine residues were added to join the CIP and the nanoglue sequences (KLHISKD-HIYPTGGGSLLGRMKGA; JG24), mimicking the original display of the KLHISKDHIYPT in the gp3 of M13 phages, in order to increase the flexibility of the display. The main advantage of the nanoglue peptide is that it interacts specifically with the spikes of the tHBcAg nanoparticles. This interaction brings the peptide and the tHBcAg very close to each other in order for the zero-length cross-linker, EDC, and sulfo-NHS to cross-link the acid groups (Asp or Glu) to amines (Lys) that interact with each other. In the absence of the nanoglue peptide, the CIP containing a Lys residue might be cross-linked to any of the Asp or Glu in the tHBcAg nanoparticles nonspecifically. This method is advantageous over the conventional way, as it involves no molecular cloning technique for the fusion of CIP to HBcAg. In addition, the tertiary and quaternary structures of the nanoparticles are preserved.

The Lys of the nanoglue (GGGSLLGRMKGA) was shown to interact with either the Asp-64, Glu-77, or Asp-78 of HBcAg by using chemical cross-linking and mass spectrometry.[37] Böttcher and colleagues[36] demonstrated that the nanoglue binds to the HBcAg dimer which constitutes the spikes of HBcAg nanoparticles. In the present study, about 30% of HBcAg monomers were cross-linked. This is likely due to the

presence of different configurations of dimers in the $T = 3$ and $T = 4$ nanoparticles.[37] Some of these configurations may have low affinity for the JG24 peptide. However, most importantly, the nanoglue (GGGSLLGRMKGA) with 14 extra amino acid residues (KLHISKDHIYPTGG) added at its N-terminal end does not impair the nanoglue's ability to bind and cross-link to about 30% of the tHBcAg nanoparticles.

Virus nanoparticles, in comparison to synthetic nanoparticles, have been shown to possess beneficial properties for medical applications in drug encapsulation and gene delivery. They can be manufactured easily in large scale, uniform in size and shape, and with high capsid cavity, and they can be modified genetically and chemically.[44] The tHBcAg used in this study possesses two lysine residues at position 7 (Lys-7) and position 96 (Lys-96). Here, we demonstrated that these residues can be conjugated with fluorescein molecules by using an amine reactive reagent. These conjugation sites can serve as external linkages to carry therapeutic drugs, such as doxorubicin and hygromicin, into cells.[45,46] In addition, by using the encapsidation procedure as described by Lee and Tan,[15] fluorescent oligonucleotides were packaged into tHBcAg nanoparticles. When the tHBcAg nanoparticles either loaded with fluorescent oligonucleotides or conjugated with fluorescein molecules were cross-linked with the CIP via the nanoglue, both the cargoes were delivered successfully into HeLa cells *in vitro*.

CONCLUSIONS

Our study demonstrated a proof of concept for displaying CIPs on HBV nanoparticles via the "nanoglue" (Scheme 2). The cross-linking of the "nanoglue" harboring the CIP had no adverse impact on the morphology of the nanoparticles. The nanoparticles displaying the CIP either labeled with fluorescein molecules or packaged with a fluorescent cargo were demonstrated to internalize HeLa cells. The "nanoglue" concept could be advantageously used to link any CIPs to protein nanoparticles for drug or gene delivery into cells.

ASSOCIATED CONTENT

Supporting Information

The methodology and results showing that fixation of HeLa cells with paraformaldehyde for fluorescence microscopy does not contribute to staining artifact, the CIP (KLHISKDHIYPT) does not cross-link to the tHBcAg nanoparticles, and localization of the tHBcAg in HeLa cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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