Virus Engineering Hot Paper

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Site-Specific Engineering of Chemical Functionalities on the Surface of Live Hepatitis D Virus**

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The genetic code expansion strategy, the recently emerged pyrrolysine (Pyl)-based system in particular, has become a generally applicable method for site-specific incorporation of unnatural amino acids (UAAs) into a protein of interest in bacteria, yeast, mammalian cells, and even in animals.[1] However, this technique has yet to be applied to intact and live viruses, which is largely due to the fragile nature as well as the complicated assembly process of many human viruses. To address this challenge, we here coupled the genetic-code expansion strategy with an engineered virus assembly process in human hepatocytes to site-specifically introduce unnatural chemical groups onto virus surface proteins by using hepatitis D virus (HDV) as a model system.

HDV has infected more than 15 million people worldwide, and currently there are no drugs clinically available against this virus. HDV is a satellite virus of human hepatitis B virus (HBV), which has infected two billion people and among them about 240 million are currently chronically infected. Both HBV and HDV share the same envelope proteins for infection of hepatocytes. Study of HBV and HDV infection has long been hampered by the lack of efficient and easily accessible in vitro infection system.[2] Recently, a bile acid transporter predominantly expressed in liver, sodium taurocholate cotransporting polypeptide (NTCP) was identified as a functional receptor for HDV and HBV.[3] The NTCP complemented human hepatoma cell line HepG2 provided a feasible in vitro infection system for studying HBV and HDV infection. However, the lack of methods to selectively label, monitor, and/or manipulate an intact virus under living conditions still restricts investigations into molecular details of the infection. Many problems are due to the distinct topological features of the critical viral proteins, as well as complex virus assembly processes.[4] For example, HDV has developed a tightly regulated assembly process to produce infectious viral particles in human hepatocytes: the HDV RNAs were first encapsulated with delta antigens and then packaged with three HBV envelope proteins, namely large (L), middle (M), and small (S) proteins, to produce the intact viral particle before being secreted to the extracellular space (Supporting Information, Figure S1).[5] The resulting HDV, with a diameter of 36 nm, is one of the smallest animal viruses known to date.[5c,6] It is therefore exceedingly difficult to chemically label this tiny virus with delicate structures under living conditions. Furthermore, the virus surface envelope proteins contain many chemically active amino acids (for example, cysteine and lysine) that are essential for virus entry in host cells.[5b,7] Conjugation or modification of these natural residues will severely compromise viral infectivity. A non-invasive strategy for manipulation of living viral particles without impairment of their viability and infectivity is thus highly desired.

Bioorthogonal reactions have revolutionized our ability to label and manipulate various biomolecules and even whole cells and organisms under living conditions. As a critical step for applying such chemistry for virus labeling, several approaches have been reported for installation of bioorthogonal handles, typically in the form of UAAs into proteins from sub-viral-like particle (SVP) or intact virus.[8] For instance, site-specific or residue-specific incorporation of UAAs bearing an azide or an alkyne moiety into SVP has been demonstrated in bacterial cells by several laboratories.[8a,9] These methods allow the conjugation of SVPs with various fluorescent dyes or therapeutic reagents for biomedical or biomaterial applications.[8a,b] However, SVPs are non-infectious and not suitable for investigating virus infection mechanisms. Indeed, SVPs produced from prokaryotic cells lack posttranslational modifications, particularly on their surface envelope proteins, and therefore differ from the native SVPs. Although attempts have been made to extend some of these methods for virus production in mammalian cells, such strategies typically require the metabolic replacement of a specific type of amino acid permissive only to simple groups (for example, azide and ketone) from the entire virus proteome, which may disrupt the virion assembly process or permute the vulnerable virion structure, resulting in compromised viral infectivity.[10] Taken together, a general approach for precise labeling and manipulation of intact

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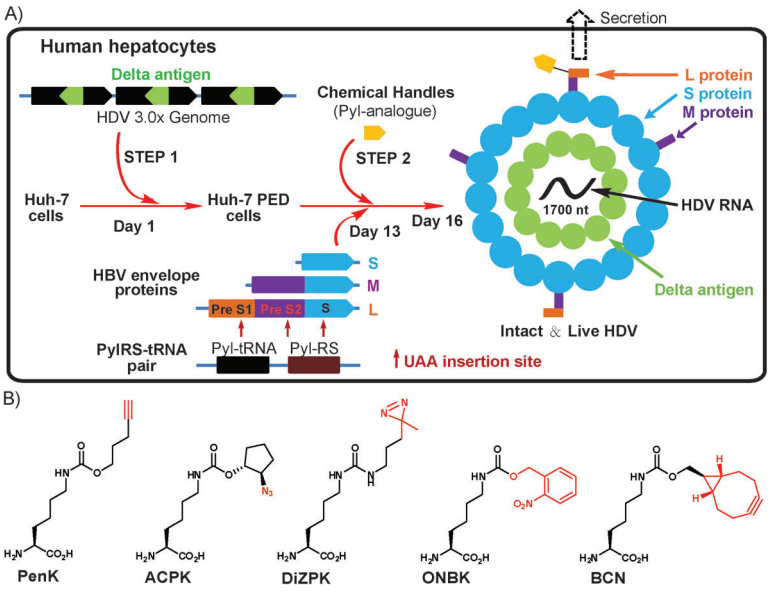
living virus with high protein selectivity and site specificity has not been fully established.

Herein, we engineered the HDV assembly process to accommodate the Pyl-based genetic code expansion strategy within viable human hepatocytes, which allowed the genetic and site-specific incorporation of Pyl analogues carrying various functional handles into the surface envelope proteins of live HDV (Scheme 1 A) with high production yield. Optimization of the UAA incorporation site and the subsequent click labeling conditions ultimately permitted the attachment of a biotin molecule onto HDV surface without compromising its viability or infectivity.

Huh-7 is a commonly used human hepatocyte cell line for producing native hepatitis virus. We started by validating the compatibility of the Pyl-based system in Huh-7 cells by using green fluorescent protein (GFP) as a model protein. As illustrated in Scheme 1 B, five recently developed Pyl analogues were employed: PenK and ACPK contain an alkyne and an azide group, respectively, which can participate the CuAAC reaction for protein click labeling;[10,11] BCN bears a cyclooctyne moiety capable of participating the inverse electron-demand Diels–Alder reaction and the 1,3-dipolar cycloaddition on proteins;[12] DiZPK possesses a photo-reactive diazirine for protein photocrosslinking;[13] and the lysine derivative ONBK carries a photolytically removable *O*-nitrobenzyloxycarbonyl group.[14] These five Pyl analogues can be effectively recognized by wild-type (WT) or mutant PylRS (Supporting Information, Table S1). Co-transfection of a plasmid encoding the corresponding PylRS variant and $tRNA^{Pyl}_{CUA}$ with a plasmid encoding GFP carrying an amber mutation at residue 40 (GFP-Y40-TAG) in Huh-7 cells successfully generated full-length GFP in the presence of any of these five UAAs, whereas no fluorescence was observed in the absence of UAAs or the PylRS-$tRNA^{Pyl}_{CUA}$ pair (Figure 1 A; Supporting Information, Figure S2). Furthermore, the integrity of these UAAs incorporated GFP was verified by mass spectrometry analysis (Supporting Information, Figures S3–S7). We then went on to test the incorporation of these UAAs into residues located at Pre S1, Pre S2, or S domain on HBV L protein (Scheme 1 A). Immunoblotting analysis confirmed that all of these five UAAs can be incorporated into L protein by their corresponding PylRS variant and $tRNA^{Pyl}_{CUA}$, and the incorporation efficiency was varied depending on the specific incorporation site (Figure 1 B).

Next, before moving to live virus, we tested the production of HBV/HDV SVP carrying a site-specifically incorporated PenK capable of bioorthogonal labeling. The SVP, with a diameter about 22 nm, is secreted in large excess (1000- to 100 000-fold excess) than the intact virus and are easier to handle with.[15] By incorporating PenK at the residue S280 on S protein, a large amount of SVP, termed SVP-S280PenK, were assembled and secreted from Huh-7 cells, whereas no SVP was detected without the addition of PenK or the PylRS-



Scheme 1. Engineering HDV assembly process for site-specific incorporation of UAAs into its surface envelope proteins. A) A two-step procedure for assembly of intact HDV carrying a site-specifically incorporated UAA in human hepatocytes Huh-7 cells: At day 1, a plasmid carrying HDV 3.0x genome was first transfected into Huh-7 cells to produce HDV RNAs and delta antigens: At day 13, the resulting cells, termed Huh7-PED cells, were transfected with the plasmids encoding HBV envelope proteins and the PylRS-$tRNA^{Pyl}_{CUA}$ pair in the presence of Pyl analogues. The intact HDV virions were collected from the cell culture medium on day 3 and 6 post-transfection. B) Structures of five Pyl analogues used in this study: PenK (N^{ε}-pent-4-ynyloxy-carbonyl-L-lysine), ACPK (N^{ε}-((1*R*,2*R*)-2-azido-cyclopentyl oxy-carbonyl)-L-lysine), BCN (bicyclo[6.1.0]non-4-yn-9-ylmethanol), DiZPK ((3-(3-methyl-3*H*-diazirine-3-yl)-propamidocarbonyl-N^{ε}-L-lysine), and ONBK (o-nitrobenzyloxy carbonyl-N^{ε}-lysine). The functional groups are colored in red.

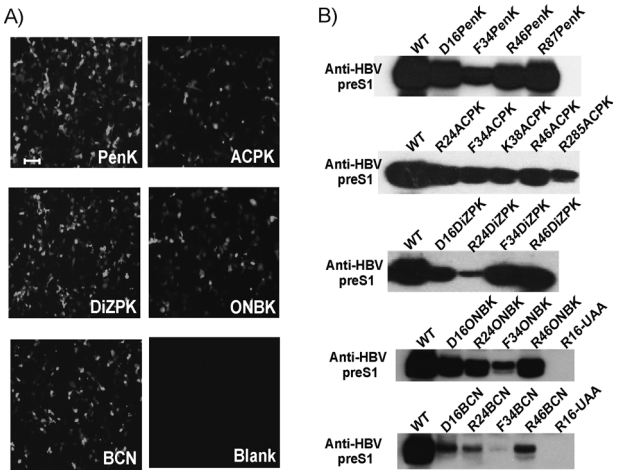


Figure 1. Encoding UAAs in human hepatocytes by the Pyl-based system. A) Huh-7 cells transfected with plasmids encoding the PylRS-$tRNA^{Pyl}_{CUA}$ pair and GFP-Y40-TAG with and without the indicated Pyl analogues (1 mM final concentration) were visualized by fluorescent microscopy. All images are in the same scale (scale bar: 100 μm). B) Western blotting analysis using the mouse monoclonal antibody-2D3 that specifically recognizes residues 19–33 of the Pre S1 domain on L protein. The expression of HBV L protein with the Pyl analogues at indicated sites in Huh-7 cells was detected by 2D3.

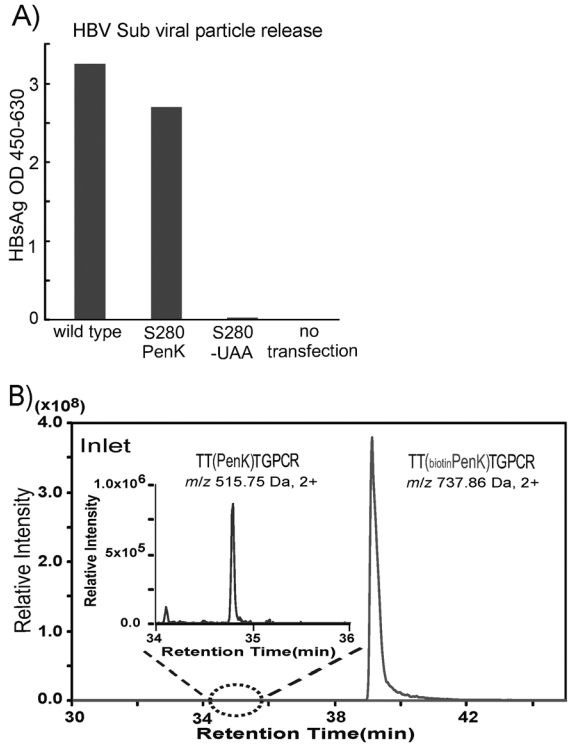


Figure 2. Site-specific installation of PenK into the HBV/HDV SVP surface. A) The secreted SVP-S280PenK from transfected Huh-7 cells was measured by an HBsAg ELISA kit. Cells transfected with a plasmid encoding WT-SVP were used as a positive control while cells without the addition of PenK or without plasmid transfection were used as negative controls. B) LC-MS/MS analysis of in gel trypsin digested sample from streptavidin pull-down SVP after conjugation azide-biotin to the surface of SVP(S277R)-S280PenK upon CuAAC (see the Supporting Information, Figure S14 for detailed information of the process). The intensity, retention time, and molecular weight of both the biotin labeled (calculated molecular weight: 515.7, 2+) and unlabeled peptide (calculated molecular weight: 737.8, 2+) are shown in the same scale. Inset: enlarged signal for the unlabeled peptide. Sequence and *m/z* of the peptides are shown above.

$tRNA^{Pyl}_{CUA}$ pair (Figure 2A). The produced SVP-S280PenK was then conjugated with an azide biotin and verified by western blotting and ELISA. The best labeling efficiency was achieved by using Cu^{I}/BPS (3-[4-({bis[(1-*tert*-butyl-1*H*-1,2,3-triazol-4-yl)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]propyl hydrogen sulfate) at a ratio of 2:1 in glove box within 30 min, which showed both glycosylated and non-glycosylated forms of S protein (Supporting Information, Figures S8–S10). Interestingly, no labeling was observed when Cu^{I}/BPS was used at a ratio of 1:2, indicating that excess amount of BPS may quench this reaction (Supporting Information, Figures S8, S9).[8a] A gel-based fluorescence assay was further used to verify the CuAAC reaction between the azide-tethered Alexa-Fluo 488 and SVP-S280PenK (Supporting Information, Figure S11), as well as the inverse-electron-demand Diels–Alder cycloaddition between the tetrazine fluorophore (5-TAMRA-X) and SVP-S280 BCN (Supporting Information, Figures S11, S12).[12,16] Finally, we examined the biotin-labeled and enriched SVP by mass spectrometry. A Serine to Arginine mutation was created at residue 277 on S protein to produce SVP(S277R)-S280PenK, which allowed us to obtain a small peptide between residues T278 and R285 that is suitable for mass-spectrometric analysis upon trypsinization. The BPS-assisted CuAAC between SVP(S277R)-S280PenK and azide biotin reached a full conversion (> 99%), as seen by the intensity of the biotin-label peptide that was more than 400 times higher than the unlabeled peptide (Figure 2B; Supporting Information, Figures S13–S16).

Encouraged by these results, we then applied our UAA incorporation and labeling strategy to live HDV particles. The accumulation of HDV RNA and delta antigen will reach a steady stage in about 10 days after the transfection of plasmids containing HDV cDNA into Huh-7 cells. However, the UAA-containing HBV envelope proteins are typically expressed and remain stable within the first 3 days post-transfection. To take full advantage of these features, we developed a two-step procedure to integrate the genetic-code expansion strategy into the assembly process of HDV in Huh-7 cells (Scheme 1A): on day 1 post-seeding, a plasmid carrying HDV 3.0× genome was first transfected into Huh-7 cells for producing high levels of HDV RNAs and delta antigens; on day 13, the resulting cells, termed Huh7-PED cells, were then transfected with plasmids encoding HBV envelope proteins and the PylRS-$tRNA^{Pyl}_{CUA}$ pair. The cells were further incubated in the presence of 1 mM Pyl analogue before harvesting the produced intact HDV at day 3 and 6 post-transfection. By systematic optimization of the ratios among these plasmids being transfected, we successfully assembled live and intact HDV from Huh-7 cells carrying PenK at residue F34 on L protein at a yield comparable to that of WT-HDV. A modest yield was achieved by incorporation PenK at residue R87. By contrast, installation of PenK at S280 significantly reduced the virus production yield (Figure 3A). We then examined the infectivity of the produced HDV variants by using HBV/HDV receptor NTCP complemented HepG2-NTCP infection system. The HDV infections were first quantified by real time RT-PCR. HDV RNA levels in HepG2-NTCP cells inoculated with HDV-F34PenK was comparable to that of WT-HDV (Figure 3B). The high infectivity of the UAA-bearing virus was further confirmed by staining the intracellular delta antigen accumulated in the infected cells 8 days after infection (Figure 3C), which could be inhibited by viral entry inhibitors (for example, anti-HBsAg mAb-17B9, anti-preS1 mAb-2D3, and HBV Pre S1 peptide-myr59) in a similar fashion as WT-HDV (Supporting Information, Figure S17).

Notably, although HDV variants carrying PenK at several additional sites (for example, T95, L120) on its surface L protein also showed similar infectivity as WT-HDV (Supporting Information, Figure S18), incorporation of PenK at the S280 site on the surface S protein dramatically reduced its infectivity (Figure 3B,C), which indicated that the HDV production is limited by the expression level of S protein. Alternatively, certain residues in S protein such as S280 have a lower tolerance with chemical modifications. Notably, the infectivity of HDV carrying a site-specifically incorporated BCN was much lower than that of HDV bearing PenK at the same site, which may be attributed to the cytotoxicity during

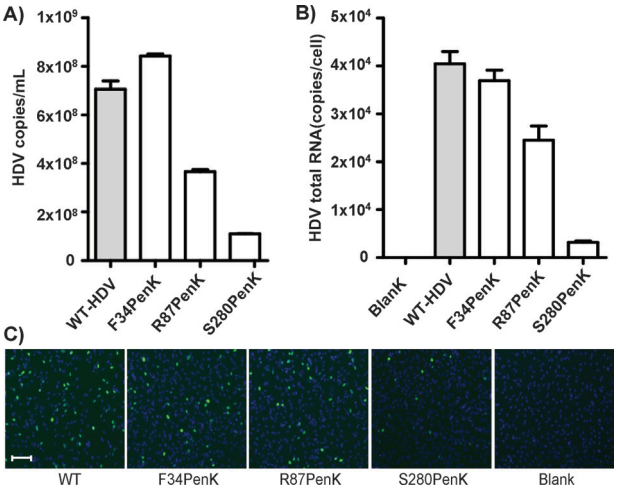


Figure 3. Site-specific incorporation of PenK into live HDV. A) The WT- and PenK-bearing HDV were collected from culture medium of transfected Huh-7 cells at day 3 and day 6 post-transfection. The HDV RNAs were isolated from cell lysate and the viral copy numbers were determined by real-time RT-PCR. Error bars represent ±s.d. of three independent experiments. B) HepG2-NTCP cells were maintained in PMM and infected by WT- or PenK-bearing HDV. At day 8 post-infection, intracellular HDV RNA was quantified by real-time RT-PCR. Error bars represent ±s.d. of three independent experiments. C) HepG2-NTCP cells infected by WT- or PenK-bearing HDV were fixed by 100% methanol at day 8 post-infection. Intracellular delta antigen was stained with a FITC conjugated monoclonal antibody (FITC-4G5) against the delta antigen. All images are in the same scale (scale bar: 50 μm).

viral production (Supporting Information, Figure S19). Furthermore, the photocrosslinker DiZPK was also used to replace residues located at the potential receptor binding region in the N termini of the Pre S1 domain (residues 8–16).However, none of the HDV variants we generated exhibited infectious capability comparable to that of WT-HDV, indicating that all of these residues are essential for HDV infection. Alternatively, installation of DiZPK in a nearby region (residue R24, F34, or R46) maintained HDV infectivity (Supporting Information, Figure S20). Considering those region might also involve in viral binding, such HDV variants may be used in combine with protein photocrosslinking to identify other potential virus binding partners on human hepatocytes.

To further confirm the incorporation and surface presentation of PenK, the azide-tagged Cy3 was used to label virus-like particles bearing PenK at residue S280 (S280PenK) by a ligand-assisted CuAAC reaction. The recently developed Cu^{I} accelerating ligand BTTPS (3-(4-((bis((1-*tert*-butyl-1*H*-1,2,3-triazol-4-yl)methyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)propyl hydrogen sulfate) was used instead of BPS, which further lowered the toxicity of Cu^{I} ions to live virus as well as hepatocyte cells.[17] Upon inoculation of S280PenK, immunostaining of the S envelope protein by an Alexa Fluor 488-conjugated antibody demonstrated excellent colocalization with the azide-Cy3 fluorophore on host cell membrane (Supporting Information, Figure S21). As a control, when WT-HDV was incubated with the cells followed by immunostaining with the same antibody, only green but not red fluorescence was observed (Supporting Information, Figure S21). The uptake process was then monitored by tracing these Cy3-labeled particles, which showed internalization into HepG2-NTCP cells 12 h post-inoculation (Supporting Information, Figures S22–S24). Therefore, our fluorescent labeling procedure using the ligand-assisted CuAAC on viral surface proteins may be applied to study cellular entry process of the hepatitis D virus.[3]

Finally, we demonstrated the enrichment of HDV bearing an alkyne residue at different incorporation sites (HDV-PenK) on its surface. WT-HDV and HDV-PenK were produced in Huh-7 cells and PEG precipitation was used for concentrating and purifying the virus before being conjugated with azide-biotin by BTTPS-assisted CuAAC. To examine the labeling efficiency, the biotinylated virus were purified by a second round of PEG precipitation to remove the Cu^{I} catalyst, ligand, as well as ascorbic acid before being enriched by streptavidin C1 dynabeads (Supporting Information, Figure S25). As expected, the pull-down efficiency of these PenK bearing HDV variants was remarkably higher than the native HDV without an alkyne handle (Figure 4 A). Importantly, the HDV-PenK virus was highly infectious: more than 50% of the infectivity of biotinylated and purified HDV-L120PenK was maintained comparing to WT-HDV (Figure 4 B). The reduced infectivity of PenK bearing HDV may

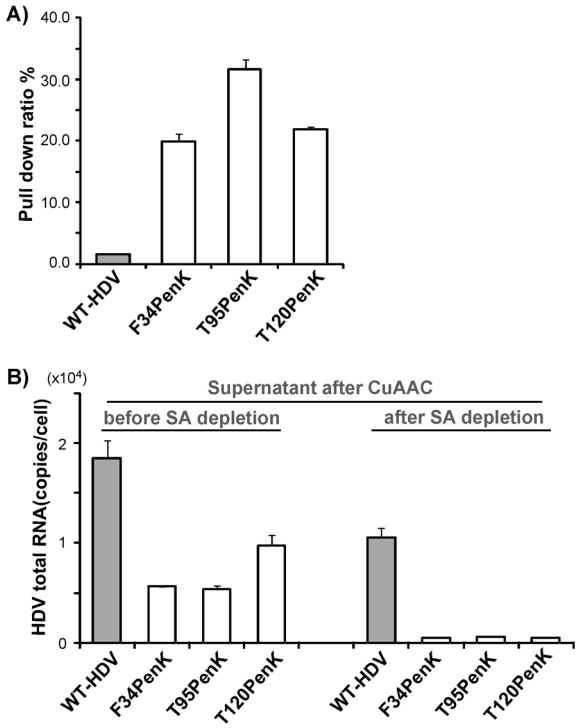


Figure 4. Bioorthogonal labeling and enrichment of live HDV with retained infectivity. A) The biotinylated HDV was pulled down by streptavidin C1 dynabeads and the HDV RNA was quantified by real-time RT-PCR. Error bars represent ±s.d. of three independent experiments. B) HepG2-NTCP cells were infected with the virus before or after being depleted by streptavidin C1 dynabeads. At day 8 post-infection, the intracellular HDV RNA was quantified by real-time RT-PCR. Error bars represent ±s.d. of three independent experiments.

be due to the damage of HDV during the labeling or purification process or the bulky biotin moiety displayed on HDV surface. The infectivity of biotinylated HDV was also confirmed by immunohistochemistry analysis on the accumulation of HDV delta antigens in HepG2-NTCP cells (Supporting Information, Figure S26). Furthermore, the integrity of the labeled virus and the specificity of our UAA labeling strategy was further demonstrated by an infection depletion experiment (Supporting Information, Figure S25). Comparing to WT-HDV, the infectivity of biotin-conjugated HDV-PenK variants were diminished after removal of the labeled virus with streptavidin beads (Figure 4B; Supporting Information, Figure S26).

In summary, by taking advantage of the unique assembly process of HDV in culture, we applied the genetic-code expansion strategy for site-specifically incorporation of UAAs beyond the canonical twenty amino acids into the surface proteins of living virus. We engineered the HDV assembly process to incorporate a panel of Pyl analogues bearing diverse functionalities into the HBV envelope protein with high site specificity and fidelity, which were assembled with the pre-generated HDV RNA and delta antigen into the intact HDV in human hepatocytes. Infectivity of these viruses can be fully maintained by optimization of the UAA incorporation site on virus surface. We further demonstrated the ligand-assisted CuAAC for selective labeling and biotin/streptavidin enrichment of live HDV without much loss of their infectivity. Owing to the fragile nature as well as the close interactions engaged between HDV and host cells, the yield and infectivity of HDV variants bearing UAA at different incorporation sites were significantly varied, which underlines the importance for site-specific engineering of chemical activities on virus surface. To our knowledge, this study provides the first example in which unnatural functionalities were site-specifically incorporated into proteins from an intact and infectious virus, and subsequently reacted with labeling probes under living conditions. Our strategy may provide a generally applicable approach for engineering and precise labeling of diverse mammalian virus that remains difficult to manipulate using conventional methods.

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[1] a) C. C. Liu, P. G. Schultz, *Annu. Rev. Biochem.* **2010**, *79*, 413 – 444; b) L. Davis, J. W. Chin, *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 168 – 182; c) A. Bianco, F. M. Townsley, S. Greiss, K. Lang, J. W. Chin, *Nat. Chem. Biol.* **2012**, *8*, 748 – 750.
[2] a) D. Lavanchy, *J. Viral. Hepatitis* **2004**, *11*, 97 – 107; b) S. Schädler, E. Hildt, *Viruses* **2009**, *1*, 185 – 209.
[3] a) H. Yan, G. Zhong, G. Xu, W. He, Z. Jing, Z. Gao, Y. Huang, Y. Qi, B. Peng, H. Wang, L. Fu, M. Song, P. Chen, W. Gao, B. Ren, Y. Sun, T. Cai, X. Feng, J. Sui, W. Li, *eLife* **2012**, *1*, e00049 – e00049; b) G. Zhong, H. Yan, H. Wang, W. He, Z. Jing, Y. Qi, L. Fu, Z. Gao, Y. Huang, G. Xu, X. Feng, J. Sui, W. Li, *J. Virol.* **2013**, *87*, 7176 – 7184.
[4] a) P. Henning, E. Lundgren, M. Carlsson, K. Frykholm, J. Johannisson, M. K. Magnusson, E. Tang, L. Franqueville, S. S. Hong, L. Lindholm, P. Boulanger, *J. Gen. Virol.* **2006**, *87*, 3151 – 3160; b) P. S. Banerjee, P. Ostapchuk, P. Hearing, I. Carrico, *J. Am. Chem. Soc.* **2010**, *132*, 13615 – 13617.
[5] a) T. S. Yeh, Y. H. W. Lee, *Virology* **1998**, *249*, 12 – 20; b) J. M. Taylor, *Virology* **2006**, *344*, 71 – 76; c) C. Sureau, *Curr. Top. Microbiol. Immunol.* **2006**, *307*, 113 – 131.
[6] S. F. Elena, J. Dopazo, R. Flores, T. O. Diener, A. Moya, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 5631 – 5634.
[7] J. Salisse, C. Sureau, *J. Virol.* **2009**, *83*, 9321 – 9328.
[8] a) E. Strable, D. E. Prasuhn, Jr., A. K. Udit, S. Brown, A. J. Link, J. T. Ngo, G. Lander, J. Quispe, C. S. Potter, B. Carragher, D. A. Tirrell, M. G. Finn, *Bioconjugate Chem.* **2008**, *19*, 866 – 875; b) L. S. Witus, M. B. Francis, *Acc. Chem. Res.* **2011**, *44*, 774 – 783; c) F. A. Rubino, Y. H. Oum, L. Rajaram, Y. Chu, I. S. Carrico, *J. Vis. Exp.* **2012**, e4246.
[9] S. S. Gupta, K. S. Raja, E. Kaltgrad, E. Strable, M. G. Finn, *Chem. Commun.* **2005**, 4315 – 4317.
[10] Z. Hao, S. Hong, X. Chen, P. R. Chen, *Acc. Chem. Res.* **2011**, *44*, 742 – 751.
[11] J. Li, S. Lin, J. Wang, S. Jia, M. Yang, Z. Hao, X. Zhang, P. R. Chen, *J. Am. Chem. Soc.* **2013**, *135*, 7330 – 7338.
[12] K. Lang, L. Davis, S. Wallace, M. Mahesh, D. J. Cox, M. L. Blackman, J. M. Fox, J. W. Chin, *J. Am. Chem. Soc.* **2012**, *134*, 10317 – 10320.
[13] M. Zhang, S. Lin, X. Song, J. Liu, Y. Fu, X. Ge, X. Fu, Z. Chang, P. R. Chen, *Nat. Chem. Biol.* **2011**, *7*, 671 – 677.
[14] P. R. Chen, D. Groff, J. Guo, W. Ou, S. Cellitti, B. H. Geierstanger, P. G. Schultz, *Angew. Chem.* **2009**, *121*, 4112 – 4115; *Angew. Chem. Int. Ed.* **2009**, *48*, 4052 – 4055.
[15] D. Ganem, A. M. Prince, *N. Engl. J. Med.* **2004**, *350*, 1118 – 1129.
[16] K. Lang, L. Davis, J. Torres-Kolbus, C. Chou, A. Deiters, J. W. Chin, *Nat. Chem.* **2012**, *4*, 298 – 304.
[17] a) W. Wang, S. Hong, A. Tran, H. Jiang, R. Triano, Y. Liu, X. Chen, P. Wu, *Chem. Asian J.* **2011**, *6*, 2796 – 2802; b) D. C. Kennedy, C. S. McKay, M. C. B. Legault, D. C. Danielson, J. A. Blake, A. F. Pegoraro, A. Stolow, Z. Mester, J. P. Pezacki, *J. Am. Chem. Soc.* **2011**, *133*, 17993 – 18001.

