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REVIEW ARTICLE

Visualization of hepatitis B virus entry – novel tools and approaches to directly follow virus entry into hepatocytes

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Hepatitis B virus (HBV) is a widespread human pathogen, responsible for chronic infections of ca. 240 million people worldwide. Until recently, the entry pathway of HBV into hepatocytes was only partially understood. The identification of human sodium taurocholate cotransporting polypeptide (NTCP) as a *bona fide* receptor of HBV has provided us with new tools to investigate this pathway in more details. Combined with advances in virus visualization techniques, approaches to directly visualize HBV cell attachment, NTCP interaction, virion internalization and intracellular transport are now becoming feasible. This review summarizes our current understanding of how HBV specifically enters hepatocytes, and describes possible visualization strategies applicable for a deeper understanding of the underlying cell biological processes.

Keywords: HBV entry; NTCP; visualization

Hepatitis B virus (HBV) infection remains a major public health problem worldwide. Although vaccines have been available since the 1980s, 240 million people are still chronically infected with HBV, many of them living in Asia or sub-Saharan Africa [1]. The therapeutic options are limited and no curative regimens are yet available. About 15–20 million people are superinfected with the hepatitis D virus (HDV) [2], which envelopes its viroid-like RNA-genome using the HBV envelope proteins to disseminate. For many viruses, entry plays an important role in determining cell tropism and species specificity [3]. Until recently, entry of HBV has not been well understood, mostly because of the lack of efficient and convenient *in vitro* infection systems. In 2012, the group of Wenhui Li identified the human sodium taurocholate cotransporting polypeptide (NTCP), a hepatocyte-specific bile salt transporter, as a functional receptor for HBV and HDV [4]. NTCP was confirmed as a receptor by Ni *et al.* [5], and subsequently efficient cell culture systems for HBV and HDV were established by overexpressing NTCP in HepG2 and HuH7 hepatoma cell lines [4,5]. These cell culture systems have partially replaced previously used primary human hepatocytes (PHH) or differentiated HepaRG cells, which are technically difficult to culture and manipulate. With these novel cell lines, we can now genetically alter NTCP, in a way that it still binds HBV/HDV but cannot fully support infection. This will allow us to discriminate between binding events and subsequent steps of infection. Moreover, since fusion of NTCP to fluorescently labelled tags or reporter-proteins like GFP neither interferes with its HBV/HDV receptor function (Y. Ni, unpublished), nor its activity as a bile salt transporter

Abbreviations
ASBT, apical sodium-dependent bile acid transporter; cccDNA, covalently closed circular DNA; DHBV, duck hepatitis B virus; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FPs, fluorescent proteins; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis D virus; HIV, human immunodeficiency virus; HSPGs, heparan sulphate proteoglycans; NTCP, human sodium taurocholate cotransporting polypeptide; ORFs, open reading frames; PHH, primary human hepatocytes; rcDNA, relaxed circular DNA; SOAT, sodium-dependent organic anion transporter; SVPs, subviral particles; TC, tetracysteine; VSV, vesicular stomatitis virus.

[6,7], labelled NTCP can be used for visualization purposes.

During the past years, advances in fluorescence labelling techniques of viral particles and peptidic ligands have been achieved. In addition, fluorescence microscopy with high sensitivity and spatiotemporal resolution has evolved as a powerful tool to unveil and follow single virus particles during entry. Many fluorophores like fluorescent proteins (FPs) and chemical dyes are available, and multiple labelling strategies have been developed. Such techniques have been successfully applied to study the entry of viruses such as human immunodeficiency virus (HIV) and influenza virus [8,9]. For several reasons, it is difficult to apply these techniques for HBV. Firstly, HBV has a small and tightly organized genome, where nearly every nucleotide is protein encoding with heavily overlapping open reading frames (ORFs) [10] (Fig. 1B). Furthermore a size limitation of 3.5 kb [11,12], which restricts the direct incorporation of marker genes into the HBV genome, has been described. Secondly, the purification of HB virions is challenging because of the presence of a huge excess (up to 10 000-fold) of so-called subviral particles (SVPs), which do not contain HBV genomes. Although several reports attempted to fluorescently label HBV and its SVPs [13–18], more investigations are needed to optimize the labelling strategies and develop methods that allow separation of virions from SVPs. Due to these restrictions, approaches aiming at specifically labelling of single amino acids (e.g. by click chemistry) become especially attractive for HBV and the related HDV.

Here, we summarize our current view of HBV entry and describe possible visualization techniques to reveal the mechanism of HBV entry.

Current understanding of HBV entry mechanism

Attachment to heparan sulphate proteoglycans as a prerequisite to NTCP interaction

HBV entry into hepatocytes requires low specificity interaction with heparan sulphate proteoglycans (HSPGs), most likely prior to the highly specific NTCP receptor interaction (Fig. 2). This is based on the observation that HBV and HDV infection of NTCP-expressing PHH and HepaRG cells can be completely blocked by heparin and other highly sulphated molecules [19]. Moreover, HepaRG cells, when treated with sodium chlorate, become deficient in HSPG formation and refractory to HBV [20]. HSPG are abundant on the cell surface and show a large diversity regarding the degree of sulphation. As HBV attachment can be preferentially blocked by highly sulphated inhibitors such as heparin and dextran sulphate, but not by the less sulphated chondroitin sulphate [19,21], HBV is thought to preferentially enrich at the highly sulphated HSPGs on hepatocytes and to a lower degree at those expressed on endothelial and dermal cells [22]. The recent observation of a preference to bind Glypican 5 [23], which is highly expressed in the liver [24], may partly explain the hepatotropism of HBV. Nevertheless, it remains unclear, how the virus efficiently infects chimpanzees *in vivo* without being unproductively sequestered by nonhepatic cells before reaching the liver. One explanation is the finding that virions exist in two clearly distinct configurations [25], which differ in their capability to bind HSPGs [26]. Binding of HBV to HSPGs on parenchymal liver cells leads to the enrichment of HBV at the cell membrane and triggers high-affinity binding to NTCP [27].

HBV envelope proteins and specific binding to the NTCP receptor

The HBV and HDV envelopes contain three different proteins called large (L), middle (M) and small (S) surface protein (Fig. 1). The three proteins share the C-terminal S domain, which on its own forms 22 nm spherical subviral particles (spheres). The M protein contains an extra preS2 domain N-terminal to S and the L protein harbours the additional preS1 and preS2 domains [3,28]. L- and S-proteins contain determinants which are crucial for viral entry of HBV and HDV [29]. Early studies demonstrated that the preS1 domain of the L protein is required for viral infectivity [30,31]. Consistent with these observations, N-terminally myristoylated peptides containing N-terminal amino acids of the preS1 domain were shown to potently inhibit viral infection of PHH or tupaia hepatocytes or of cultures of differentiated HepaRG cells, likely by targeting a cellular receptor for the virus [32–34]. A lead compound of such peptides comprising the N-terminal 47 amino acids (Myrcludex B) is presently in clinical development as an entry inhibitor [35].

Over the past 30 years, a number of host factors have been reported to function as putative HBV receptors. They have been identified mostly by binding assays using HBV particles or recombinant envelope proteins [3,36]. None of these factors have been shown to render nonsusceptible hepatoma cells susceptible to HBV infection. In 2012, Yan *et al.* [4] applied an inhibitory myristoylated preS-peptide for a photo-

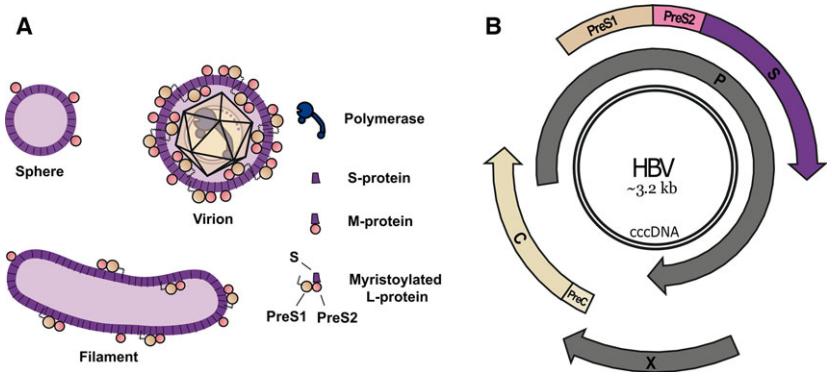

Fig. 1. (A) Schematic image of hepatitis B virus particles. In the middle, the hepatitis B virion is displayed with an incorporated DNA-containing nucleocapsid. On the left, the two types of subviral particles, spheres and filaments, are shown. The envelope of the three particle types is composed of the small (S), middle (M) and the myristoylated large (L) proteins that are depicted separately on the right. All three share the same C-terminal S domain. In addition, the M and L protein contain an N-terminal extension, the preS2-domain. The L protein encompasses a further N-terminal preS1-domain. (B) Genome organization of HBV. The two black lines represent the double-stranded covalently closed circular DNA (cccDNA), which serves as a genomic template in infected cells. The overlapping open reading frames (ORFs) for HBx (X), HBc and HBe (preC/C), the HBV envelope proteins (preS/S) and the viral polymerase (P) are shown. Note the high degree of overlapping ORFs restricting the insertion of foreign marker genes.

crosslinking, tandem affinity purification and mass spectrometry approach and identified NTCP as a receptor for both HBV and its satellite virus HDV. They characterized the interaction between NTCP and an inhibitory preS1-derived lipopeptide and proved the indispensable role of NTCP for the susceptibility to HBV/HDV infection. Importantly, ectopic expression of tupaia Ntcp and human NTCP in nonpermissive HepG2 and HuH-7 hepatoma cells rendered these cells susceptible to HBV and HDV infection. These findings have been confirmed through an independent approach by Ni *et al.* [5] and by several other studies [37–39]. NTCP is encoded by the SLC10A1 gene in humans [40]. One important function of NTCP is the sodium-dependent uptake of conjugated bile salts from the blood in the enterohepatic cycle of bile acids [40,41]. NTCP is localized at the sinusoidal membrane of hepatocytes. Its expression is highly liver-specific and regulated by effectors like bile acids, cytokines, and hormones [42]. These features correlate with the pronounced tissue specificity of HBV infection. NTCP-complemented human hepatoma cells such as $HepG2^{NTCP}$ cells are now being used as a convenient cell culture system for drug screening and for the research of HBV entry, including the visualization of the entry processes of viruses and peptidic viral ligands.

Studies on the HBV receptor function of Ntcps from different species led to the identification of separate sequence elements within human NTCP, which are crucial for HBV binding and infection. (a) Ntcp of cynomolgus monkey neither supports HBV and HDV infection nor shows binding to myristoylated preS-peptides [4,5]. The fact that such peptides cannot bind to cynomolgus hepatocytes and are devoid of enriching in the liver of cynomolgus monkeys has been previously demonstrated [43,44]. On the molecular level, amino acids 157–165 of human NTCP are crucial for the binding to preS1 [5,45]. (b) Ntcps from mouse and rat can bind the peptides (and probably also the virus) but are unable to promote HBV or HDV infection [46]. Interestingly, another sequence motif (amino acids 84–87) of human NTCP is crucial to render the binding-competent mouse Ntcp susceptible to HBV infection [5,45]. Furthermore, HBV and HDV infections depend exclusively on NTCP, as other bile acid transporters, e.g. the apical sodium-dependent bile acid transporter (ASBT) and sodium-dependent organic anion transporter (SOAT), neither bind to Myrcludex B nor support HBV infection [38].

Endocytosis and membrane fusion

HBV entry into hepatocytes probably depends on endocytic processes involving different host factors. Caveolin and clathrin have been described to participate in HBV entry into HepaRG cells and Hus-E/2 immortalized PHH [47,48]. In addition, disruption of tight junctions between HepaRG cells was demonstrated to increase HBV infection, suggesting that the entry of HBV into hepatocytes occurs at the basolateral membrane and requires cell polarization [49].

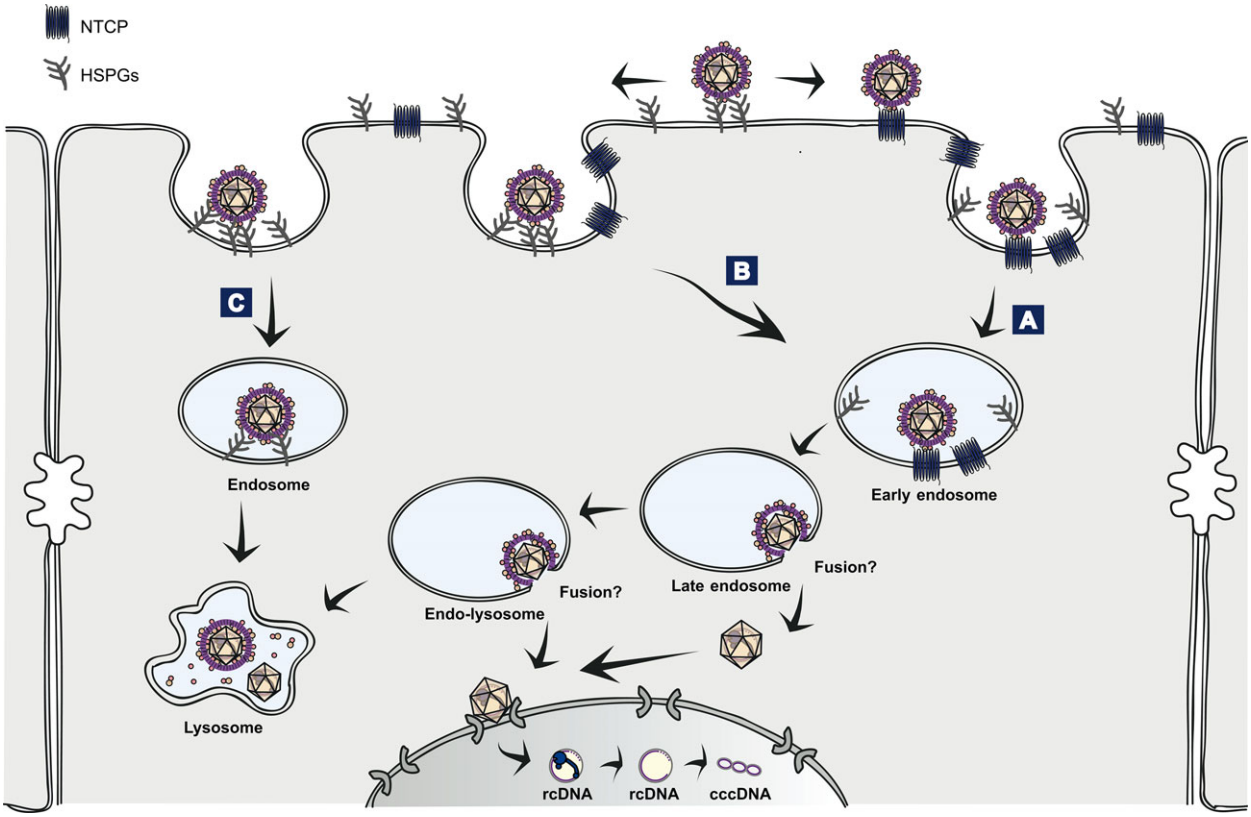

Fig. 2. Early infection steps of the HBV life cycle. HBV binds to heparan sulphate proteoglycans (HSPGs) on the cell surface [21]. Subsequent binding to NTCP has been shown to be essential to establish a productive infection [4,5,37–39]. It remains to be determined whether HBV uptake/endocytosis is NTCP-dependent (A) or mediated by HSPGs followed by subsequent interaction with NTCP inside an endosomal compartment (B) or both. In addition to an NTCP-dependent uptake, an uptake in the absence of NTCP can be hypothesized, leading to degradation or release from the cell without leading to a productive infection (C). This could play a role especially when using artificial *in vitro* infection systems. Rab5 and Rab7 have been reported to be involved in HBV infection [56], indicating that HBV has to be transported from early to mature endosomes. A partial dependency on both caveola- and clathrin-mediated endocytosis has also been described [47,48].

Most enveloped viruses that use endocytosis to deliver their genomes rely on pH-dependent processes in order to trigger fusion of the vesicular and viral membrane. However, ammonium chloride, which raises the endosomal pH, did not interfere with the infection of duck hepatitis B virus (DHBV), a model system that has been previously used to study hepadnaviral infection [50,51]. Bafilomycin A1, which neutralizes the luminal pH of the endosomes [52] and interferes with formation and maturation of endosomal carrier vesicles from early endosomes [53], inhibited both DHBV [54] and HBV [55] infection, presumably by blocking the transport and sorting of the endocytosed viral particles [51]. A recent study reported that HBV infection of HepaRG cells depends on Rab5 and Rab7 [56], which are GTPases involved in the endosome biogenesis [57]. These findings support the hypothesis that HBV is transported from early to mature endosomes. However, these initial findings have to be confirmed in other infection systems (including PHH) and should involve comparative studies with HDV that also requires HSPGs and NTCP as receptors.

Viral fusion involves multiple sequential protein–protein interactions between viral fusion proteins and host receptors, which result in conformational changes of the envelope proteins [58]. However, no studies have yet been undertaken in order to elucidate whether the conformation of the HBV envelope changes upon interaction with HSPGs or NTCP. Thus, the molecular mechanism of membrane fusion of HBV remains unclear. The Na^+ gradient and the inward Na^+ current are dispensable for NTCP-mediated HBV entry [59]. Therefore, HBV is unlikely to be coupled to the sodium gradient to drive conformational change of the receptor. Whether binding of the virus or the peptidic preS-ligand induces endocytosis of NTCP remains to be determined. Furthermore, it is unknown if the virus requires NTCP-mediated endocytosis or whether

HSPGs can promote this process and NTCP mediates productive entry only inside the endosomal compartment or both (Fig. 2). Studies revealed the microfilament- and microtubule-based traffic of NTCP between the cell surface and an intracellular vesicular pool [60,61]. A recent report showed that the epidermal growth factor (EGF) receptor (EGFR) cotraffics with NTCP into common endocytic vesicles where they segregate, with EGF-EGFR trafficking to lysosomes and NTCP being recycled to the plasma membrane [62]. These studies imply a possible function of EGF-signalling pathways in the intracellular trafficking of NTCP and consequently HBV. Furthermore, the compartment of membrane fusion is unknown. As a substantial amount of viral particles were traced to late endosomes and endolysosomes after infection, membrane fusion may occur in these compartments (Fig. 2) [19,21]. As the *in vitro* infection efficiency is very low, it is likely that some internalized virions are transported to the lysosome and degraded (Fig. 2).

Although HepG2^{NTCP} cells, differentiated HepaRG cells and PHH support HBV infection, the infection efficiency is relatively low and requires high multiplicities of genome equivalents to get reasonable numbers of infected cells. Addition of polyethylene glycol (PEG) 8000 and DMSO in the medium substantially increases the infection rates. Enhancement by PEG is probably associated with enrichment of virus at the cell surface [19]. The enhancing effect of DMSO is unclear and might either be associated with NTCP-associated steps (e.g. acceleration of trafficking) or might be NTCP-independent. Arguments for the involvement of NTCP-independent cellular factors came from experiments with mouse hepatocytes overexpressing NTCP. The cells do not support HBV infection although they allow entry of HDV [5,63]. Recent fusion experiments using replication-supporting but noninfectable HepG2 cells and nonpermissive mouse hepatocytes overexpressing human NTCP showed that the heterokaryonic cells fully support HBV replication, implying that these mouse hepatocytes require supplementation with (a) dependency factor(s) [46]. Interestingly, a recent report identified a mouse liver cell line (AML12), which became susceptible to HBV upon human NTCP expression, indicating that the required additional host factor(s) is (are) not strictly species specific [64].

Intracellular transport of HBV nucleocapsids to the nucleus

After membrane fusion, the incoming nucleocapsid containing the relaxed circular DNA (rcDNA) genome has to be transported to the nuclear pore complex and release its genome into the nucleus. In the nucleus, the rcDNA forms a covalently closed circular DNA (cccDNA) which serves as the transcriptional template for HBV antigens (Fig. 2). The precise location and timing of nucleocapsid release remains unknown. Rabe *et al*. introduced HBV nucleocapsids into nonsusceptible but replication competent HuH-7 cells using a microinjection method [65] or a lipid-based delivery system [66] and investigated the transportation of the nucleocapsids. Accumulation of genomic DNA could be observed in the nuclei 1 h post lipofection, indicating that intracellular transport of HBV capsid is rapid. Treatment of cells with actin-depolymerizing drugs like cytochalasin D or latrunculin B did not significantly impair the transportation of nucleocapsids, while treatment with microtubule-depolymerizing agent nocodazole prevented the process. This implicates that transportation of nucleocapsids is mainly microtubule-dependent [66]. Although these observations point at the possible mechanism of nucleocapsid transport, they have to be verified using conditions that are closer to *bona fide* HBV infection.

The HBV core protein contains a nuclear localization signal and importin β-binding motifs in its C-terminal domain [67–69], which is oriented towards the interior in immature RNA containing capsids [70,71]. Phosphorylation of the C-terminal domain could impair its interaction with RNA and enable accessibility to importins [69]. There are more than three phosphorylation sites at the C-terminal domain of the core protein [72]. However, the extent and the dynamics of capsid phosphorylation during HBV entry are unclear. Nup153, a component of the nuclear pore complex, has been shown to interact with the HBV core protein at high affinity, arresting the capsids in the nuclear basket [73]. Dissociation of the capsid may result in saturation of Nup153 binding and could clear the way for entry of the HBV DNA and polymerase [73].

Fluorescence labelling and visualization techniques to directly follow virus entry

The direct way to unravel viral entry processes is live cell imaging of fluorescently labelled infectious viral particles. Advances in fluorescent labelling techniques and fluorescence microscopy using high sensitivity and spatiotemporal resolution now allow live imaging of virus entry at the single-particle level [8]. Multicolour labelling of both virus particles and cellular structures reveal better insights into virus–host interactions. Multicolour labelling of different viral structural proteins also allows direct visualization of disassembly events

Table 1. Virus labelling strategies.

Labelling strategy	Labelling site	Examples	HBV/HDV reports
Lipophilic dyes (DiD, DiL, DiO, etc.)	Envelope	IAV [95], VSV [96], HIV [97], HCV [98]	HBsAg particles [15]
Nonspecific covalent protein labelling (hydrazine, NHS, maleimide, etc.)	Membrane protein or capsid	IAV [99], VSV [86], HIV [84], PV [100]	HBsAg particles [15,16]
Genetically encoded FPs (GFP, RFP, mCherry, etc.)	Membrane protein or capsid	HSV [79], HIV [80], VSV [78]	HBV (GFP-S) [17], HBV(GFP-M) [18]
Genetically encoded tags (SNAP, HaloTag, TC tag, Sortase A, etc.)	Membrane protein or capsid	HIV [85], VSV [87], HCV [88], PrV [101]	HBV capsid [13]
Click chemistry (protein)	Membrane protein or capsid	HDV [14]	HDV [14]
Click chemistry (nucleic acid)	Genome	AdV [102]	No
Intercalating dyes (SYTO dyes, $[Ru(phen)2dppz]^{2+,}$ etc.)	Genome	PV [100], IAV [103], BV [104]	No

IAV, influenza A virus; VSV, vesicular stomatitis virus; HIV, human immunodeficiency virus; HCV, hepatitis C virus; PV, poliovirus; HSV, herpes simplex virus; PrV, pseudorabies virus; HDV, hepatitis D virus; AdV, adenovirus; BV, baculovirus; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

such as membrane fusion, capsid release and the movement of subviral particles [9]. As convenient cell culture systems for HBV and HDV infection have now been established, the contribution of virus and host factors can be evaluated by direct visualization approaches using genetically manipulated and fluorescently labelled virions or host factors.

Visualization approaches depend on feasible labelling techniques for the respective viruses. An ideal fluorescence labelling method must not impair the infectivity of the virus and the function of the host cell component. It should lead to the incorporation of strong and photostable fluorophores that allows long-term tracking. Many fluorophores have been successfully used in virus labelling, including (a) FPs, such as GFP, RFP and mCherry, (b) small fluorescent molecules, such as Cy3, Cy5 and FITC and (c) big fluorophores, such as quantum dots, which are semiconductors with strong signal and long-term photo stability. Besides the traditional FPs fusion method, multiple site-specific or unspecific, covalent or noncovalent labelling strategies have also been successfully used for virus labelling (Table 1).

Applications of labelling and visualization techniques to unveil HBV entry pathway

Fluorescent labelling of NTCP and preS1 peptides

NTCP is a multi-transmembrane glycoprotein with its N terminus at the extracellular side and the C terminus at the intracellular side, according to topology studies on a related SLC10 family member [74,75]. NTCP has been successfully fused to GFP without losing its function as a bile salt transporter [38]. In addition, GFP fusion at the C terminus of human NTCP does not impair its receptor function for HBV and HDV (Y. Ni, unpublished). Insertions of a 20-kD SNAP tag at the C terminus of NTCP (NTCP-SNAP) or a 9-kD ACP tag at the N terminus (ACP-NTCP) and further labelling with the fluorescent substrates of SNAP and ACP have also been reported [7]. Both NTCP-SNAP and ACP-NTCP are functional to transport bile acids [7]. However, their ability to mediate HBV entry is unknown.

Binding of HBV and peptidic preS-ligands may alter the location and trafficking of NTCP. Understanding this process is central for understanding the mechanism of HBV entry. Therefore, binding of fluorescently labelled preS-peptides like atto-dye-labelled Myrcludex B to NTCP and elucidation of trafficking of this complex will provide insights into how HBV entry proceeds. Subsequently, the use of fluorescently labelled HBV and HDV particles in appropriate cell lines will help to clarify the pathways used by the viruses to productively enter cells.

Challenges for labelling and purification of HBV

Because of the tight organization and size limitation of the HBV genome [10–12] (Fig. 1B), incorporation of a complete reporter gene (e.g. GFP) and simultaneous maintenance of replication competence are very unlikely. From a structural point of view, the HB virion is densely packed with the capsid and envelope proteins in close contact forming multiple interactions. Integration of large tags into the capsid therefore probably interferes with virion assembly. An HB virion that encodes a directly detectable tag and still remains infection competent is not available up to date.

As an alternative, chemical labelling techniques may be applied. They have been used successfully for viruses such as HIV, hepatitis C virus (HCV), vesicular stomatitis virus (VSV) and influenza A virus (Table 1). As most of the chemical labelling methods are unspecific, they have to be combined with purification methods, which remove labelled nonviral contaminants. The NHS ester labelling approach and lipophilic dyes have been reported for labelling of SVPs formed by HBsAg [16]. No chemical labelling of infectious HB virions has been reported so far. Due to the large excess of SVPs produced by infected cells, any chemical labelling strategy would target both infectious virions and noninfectious SVPs. Thus, efficient purification methods have to be applied to enrich labelled virions. Established techniques like combination of PEG precipitation, ultracentrifugation through a sucrose cushion and isopycnic sucrose density gradients can be employed to achieve separation between virions, SVPs and naked nucleocapsids [76]. In addition, heparin affinity chromatography has been used to partially separate spherical SVPs and HB virions [77]. As binding to heparin depends on the presence of the preS-sequence [19], enrichment of particles with a high L protein content, such as virions and filamentous SVP, can be achieved. Heparin affinity chromatography can therefore be used to remove subviral particles with low L protein content and virtually all naked nucleocapsids in addition to nonheparin-binding proteins. Furthermore, size-exclusion chromatography on Superose-6 columns is a suitable way to separate virions from remaining spherical and short and intermediate filamentous SVPs [25]. As a complete separation of subviral particles from virions is very difficult to obtain, a final confirmation of the acquired fluorescent signal on the single particle level has to be incorporated into the analysis.

HB virions and subviral particles labelled with fluorescent proteins

FPs (e.g. GFP) have been incorporated into many viruses such as VSV [78], herpes simplex virus (HSV) [79] or HIV [80]. However, as incorporation of the *gfp* gene into the HBV genome in *cis* abrogates its replication, this approach can only lead to labelling of infected cells [11]. In order to obtain fluorescent virions for live cell imaging, fluorescent fusion proteins have to be trans-complemented with the wild-type HBV genome in order to be eventually incorporated into the particle. Lambert *et al*. [17] showed that GFP can be N-terminally added to the S protein and that this labelled S protein can be integrated into SVPs in the presence of wild-type S. When HuH7 cells are cotransfected with GFP-S and HBV DNA, GFP-labelled particles containing HBV DNA are secreted [17]. The chimeric GFP-tagged particles were able to attach to HepG2 cells. However, as HepG2 cells used in this study are not permissive for HBV infection, it remains unclear whether this binding represents a relevant step of infection. In another study, GFP was tagged to the N terminus of the M protein. Trans-complementation assays suggest that this fluorescent M protein could be incorporated into HB virions or SVPs, when wild-type M protein is absent [18]. Nevertheless, the authors could not conclusively prove that fluorescent virions were obtained, and as with the GFP-tagged S protein, infectivity of these fluorescent particles has not been verified.

As the separation of labelled SVPs from virions is challenging, an alternative could be the labelling of the nucleocapsid or the genome. Such labelling techniques would also allow to follow the trafficking of capsids to the nuclear pore complex. The structure of HBV core particles has been resolved using cryo-electron microscopy and three-dimensional image reconstruction [70,81]. In addition, it has been shown that replacement of P79 and A80 of the core protein (which form the tip of the spike, formed by two alpha helices) with an entire GFP results in capsid-like particles with GFP located at that site [82]. Unfortunately such capsids cannot be enveloped due to the steric hindrance of envelopment by the large GFP protein [83]. Still, artificial delivery of such GFP-capsids (e.g. by microinjection) may help to study intracellular trafficking events although it remains unclear if the results are valid for authentic capsid trafficking [65]. This finding may also lead to investigations aiming at the insertion of much smaller tags at that very position in order to produce infectious virions.

Chemical labelling of HB virions and SVPs

Chemical fluorophores (e.g. FITC or Texas Red) have the advantage of being smaller and more photostable than FPs and have been widely used to label HIV [84,85], VSV [86,87] and HCV [88]. New chemical fluorophores with advanced properties are continuously being developed. For example, quantum dots have been applied in live cell imaging and virus tracking. They are highly photostable and show bright fluorescence [89]. Other novel dyes have photoactivatable or photoswitchable dye properties, which are required for super-resolution microscopy technologies like STORM or PALM.

When used to label proteins, chemical dyes are typically coupled to primary amines at the N terminus and in the side chain of lysine (Lys, K) amino acid residues

by ester crosslinking with amine-reactive groups (e.g. NHS ester). In a first attempt to visualize HBV attachment to cells, SVPs and preS1 peptides were conjugated to polystyrene beads through glutaraldehyde and amine group coupling. Using this method, Paran *et al.* [90] observed attachment of the conjugated SVPs and peptides to DMSO-treated HepG2 cells. The attachment of HBV envelope proteins was evaluated at single-cell resolution and it was confirmed that regions in both the S and the preS1 region contributed to attachment [90]. In another study, Hao and colleagues labelled S protein in subviral particles with a Cy5-coupled NHS ester dye. They showed that these labelled SVPs could be taken up by COS-7 cells. Disruption of the formation of clathrin-coated vesicles did not affect endocytosis of such SVPs, whereas blocking caveolin-dependent endocytic pathways by depletion of cholesterol reduced uptake of the particles [15]. Tracking of individual particles in living cells suggested that movement of SVPs within the cell depends on actin and not on microtubules. Colocalization of the labelled SVPs with GFP-tagged actin fibres supported this hypothesis [15,16].

An improvement in chemical labelling techniques addressing reactive side chains of amino acids is the incorporation of peptide tags to target proteins which can then be specifically labelled with a fluorescent dye. One well characterized small tag is the 6-amino acid tetracysteine (TC) tag, which can be labelled with biarsenical dyes (FLAsH). Successful visualization using the TC tag has been shown for HIV [91], alphavirus [92], bluetongue virus [93] and HCV [88]. For HBV, the TC tag has been incorporated into the core protein, and virus particles labelled with a biarsenical dye were secreted into the medium [13]. Like the labelled SVPs described above, these labelled HBV particles could be internalized into HepG2 cells [13]. Further work is needed to prove that the TC-tagged HBV particles are infectious virions.

Labelling the core protein with small tags seems to be the most promising approach so far in order to obtain fluorescent virions for live cell imaging. Recently, amber suppression technology with minimal impairment of the target protein structure has become a promising method to fluorescently label proteins [94]. The technology employs the incorporation of single unnatural amino acids into target proteins and subsequent fluorescence labelling of the unnatural amino acid with click chemistry [94]. So far, this technique has not been applied to HBV. However, Lin *et al.* [14] used this technology to label HDV by incorporation of unconventional markable amino acids into the envelope proteins. This technology is highly promising and might also be feasible for labelling HBV envelopes or capsids.

Conclusions and perspectives

With the accessibility of novel infection systems, several aspects regarding HBV entry are about to be solved. The initial attachment to HSPGs, subsequent NTCP binding, endocytosis, membrane fusion, intracellular and nuclear transport should be investigated at temporal and spatial levels. As ideal tools, fluorescence labelling and visualization techniques have provided new opportunities. However, labelling of HBV remains to be optimized by using different strategies that target different components of HBV including envelope, capsid and genome. Successful application of labelled HBV particles in the newly established efficient infection systems will undoubtedly provide critical insights into HBV entry.

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Author contributions

All authors contributed to the writing of the manuscript.

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