IV DISCUSSION

The data of this study demonstrate the use of HBV capsids as VLPs to cover and transport foreign nucleic acids. Nucleic acids packaged in capsids are stable and resistant to treatment with DNase/RNase. To allow VLPs to overcome the outer membrane of different cell types in an endocytosis-independent manner the translocation motif - TLM was fused. TLM-mediated transfer of large aggregates such as nucleocapsids requires that the surface of these particles is coated with TLM peptides. This was achieved by fusion of the TLM to the N-terminus of HBcAg monomer, resulting in the presence of 240 TLMs on the surface of one assembled particle. The expression of recombinant capsid proteins and packaging of nucleic acids was developed in pro- and eukaryotic systems. Nucleocapsids were purified efficiently in two steps. In contrast to known VLPs, cell permeable TLM nucleocapsids translocate directly into the cytoplasm of almost all cells and bypass the endosomal compartment. Efficient gene transfer in immortalized cell lines as well as in primary human hepatocytes was achieved by using cell permeable TLM nucleocapsids.

These features would be advantageous in overcoming some of the limitations and risks of current gene transfer technologies, avoiding e.g. the low transfer efficiency and instability of naked DNA or the ability of retroviruses to transduce only dividing cells [202]. Also the risk of insertion of genes which often leads to complications observed by the use of retroviral gene transfer systems [203] can be prevented.

Furthermore it is possible to use this new system to investigate the early post entry steps of HBV infection. It was demonstrated that the TLM nucleocapsids like the HBV capsid during the infection process interact with several cytoplasmic partners. The colocalisation with the cytoskeleton and reorganization of F-actin was shown. And finally the question of structural conformation of nuclear localized HBV core protein was addressed to the TLM nucleocapsids.

IV.1 TLM MEDIATED CAPSID TRANSLOCATION

A large number of hydrophilic molecules such as peptides, proteins, and oligonucleotides are poorly internalized by cells because they are unable to cross the lipid barrier of the plasma membrane. The conventional mode of cellular entry for hydrophilic macromolecules, involves absorption to the plasma membrane or a membrane-bound receptor, followed by energy-dependent formation of a vesicle [204]. Thereafter, the endocytotic

machinery sorts the molecules into different compartments tagged for destruction or recycling. The release of molecules taken up by endosomes into the cytoplasm is rare. The endocytotic process is therefore unsuitable for the delivery of macromolecules into the cytoplasmic or nuclear compartments. This is considered to be a major limitation for the use of macromolecules as therapeutic agents in biomedical research and the pharmaceutical industry.

Cell permeability, in comparison, is an often energy-independent mechanism of peptide translocation across the cellular membrane that allows addressing of conjugated cargoes into the cytoplasm and nucleus. Cell penetration is not receptor-dependent and thus, not cell-specific. This can be a disadvantage for selective cellular targeting, but could still provide a universal system for the delivery of hydrophilic macromolecules into the cytoplasmic or nuclear compartments of any type of cell [205].

It has been reported that the use of short (10–30 amino acids) peptides may provide a solution making the intracellular delivery of such intractable molecules possible, both *in vitro* and *in vivo* [205,206]. These cell-penetrating peptides (CPPs) are either basic segments of RNA- or DNA-binding proteins [31,32] such as pAntp and Tat, or artificial peptides such as Transportan [207], model amphipathic peptide (MAP) [33], or other synthetic peptide sequences [208]. Understanding the mode of CPPs entry and defining their intracellular localization are of particular interest for the design of conjugates for cargo or drug delivery. After CPP mediated cell entry these conjugates could release membrane in-permeable, potentially instable therapeutic agents directly into the cytoplasm.

To investigate CPP passage across a membrane under realistic and physiological conditions in this study living cells were exclusively used. In contrast to artificial bilayers as another experimental system for investigation of CPP translocation the living cells provide all proteins located at or in the plasma membrane. Some membrane-proteins may be necessary for the interaction of TLM capsids with the cellular surface and for translocation. With this experimental setting it was the aim to directly examine whether the uptake of TLM capsids was related to the translocation mechanism described for others CPPs or depended on an active cellular process.

IV.1.1 Mechanism of TLM capsid translocation

Several studies of CPP translocation show that the observed uptake is merely an artifact from membrane disruption when fixing cells. The negatively charged cell membrane attracts positively charged peptides, which could lead to misinterpretations using FACS analyses. After attaching at the outer membrane the CPPs can be taken up by receptor mediated endocytosis. Disruption of the membranes and endosomes through harsh cell fixation can result in the release of peptides into the cytosol and nucleus. False positive results can lead to the assumption of a non-endocytotic translocation pathway [41,44,209]. To avoid a misinterpretation and to verify the initial findings very carefully various different methods and controls were used in this study. To exclude that the TLM capsids were not only attached to the cell surface microscopic analysis was performed by confocal laser scanning microscopy (using very small optical sections of the cell). Several optical sections were acquired. Each experiment was controlled by using the wildtype capsids with the same structure and features except for the TLM. If there was any artificial staining inside the cell, caused by fixation of the cell or by endocytotic uptake, it would have been observed in the wildtype capsid control. No staining of wildtype capsids inside the cell was observed. In addition to that the cell permeability of TLM capsids was verified by a life cell assay with fluorophore-labeled capsids. Application of capsids or nucleocapsids even for prolonged culturing times did not affect cell growth rate. During or after the treatment of cells with capsid (up to a concentration of 100 nM) no cytotoxic effect was observed.

The well controlled data of this study disprove the recently published results of Cooper and Shaul [210]. The authors demonstrated that the bacterially expressed wildtype HBV capsids built by full length HBcAg including the Arginine rich C-terminus are able to enter different cell types. These observations are based mainly on FACS analysis data. As criticized often before [41,42] by using this method it is not possible to discriminate between plasma membrane-associated and internalized proteins or macromolecules. Again, full length HBV wildtype nucleocapsids used in this study are not able to enter cells; therefore they do not show any intracellular staining or are capable to perform gene transfer. This also excludes that the C-terminus of HBcAg has the ability to mediate the translocation of the complete particle. Only the TLM is responsible for overcoming the plasma membrane.

Previous work showed that TLM mediates an energy- and receptor-independent transport across the membrane [52]. In accordance with this it

was tested whether inhibition of the respiratory chain or the endocytotic machinery affects the membrane translocation of TLM nucleocapsids (**Figure 27**). In order to exclude inhibitor-dependent artifacts on cellular translocation, experiments were conducted with ammonium chloride, chloroquine and sodium azide known compounds which interfere with the process of endocytosis by different mechanisms. The confocal microscopy data demonstrate neither inhibitors of endocytosis and endosomal acidification nor depletion of ATP can interfere with the translocation of TLM nucleocapsids into the cytoplasm. Moreover, translocated nucleocapsids do not colocalize with transferrin, a marker of the endocytotic pathway. This suggests a translocation mechanism of TLM capsids which is not mediated in an energy or endocytosis manner. By decreasing the temperature the amount of TLM capsids translocating into cells was reduced. In comparison to other CPPs and their cargos the HBV derived TLM capsid has a size of about 5000 kDa with a diameter of about 30 nm. Translocation of TLM capsids via a non-endocytotic way depends on a maximum of membrane flexibility and fluidity. At low temperature the lipid tails pack closely with one another and this leads to a decreased fluidity of the membrane and translocation of TLM capsids.
In summary it can be ascertained that the results shown demonstrate impressively the ability of TLM capsids to translocate directly into the cytosol of cells under physiological conditions.

To explain these observations, it has been proposed that the hydrophobicity and amphipathicity of CPPs confers with the ability to interact with and pass through the lipid matrix of the plasma membrane via an energy-independent process termed translocation [34,205,211]. In accordance with this model, biophysical studies have indicated that pAntp, Transportan, or MAP can interact with lipid vesicles while forming a helical structure [212-215].
Based on these predictions it is suggested that the TLMs on the surface of a capsid are able to interact in a similar way with the outer membrane of a cell. According to the modeling data (**Figure 21**) it is suggested that the exposed amphipatic α–helices of the double TLM form a hairpin-structure. As a consequence of this only the hydrophilic and positively charged parts of TLMs are exposed on the surface of capsids. This leads to a strong interaction with the phospholipids of the outer membrane. As shown in **Figure 44** after the first interaction, the double TLM hairpin–structure is able to unfold inside of the membrane.

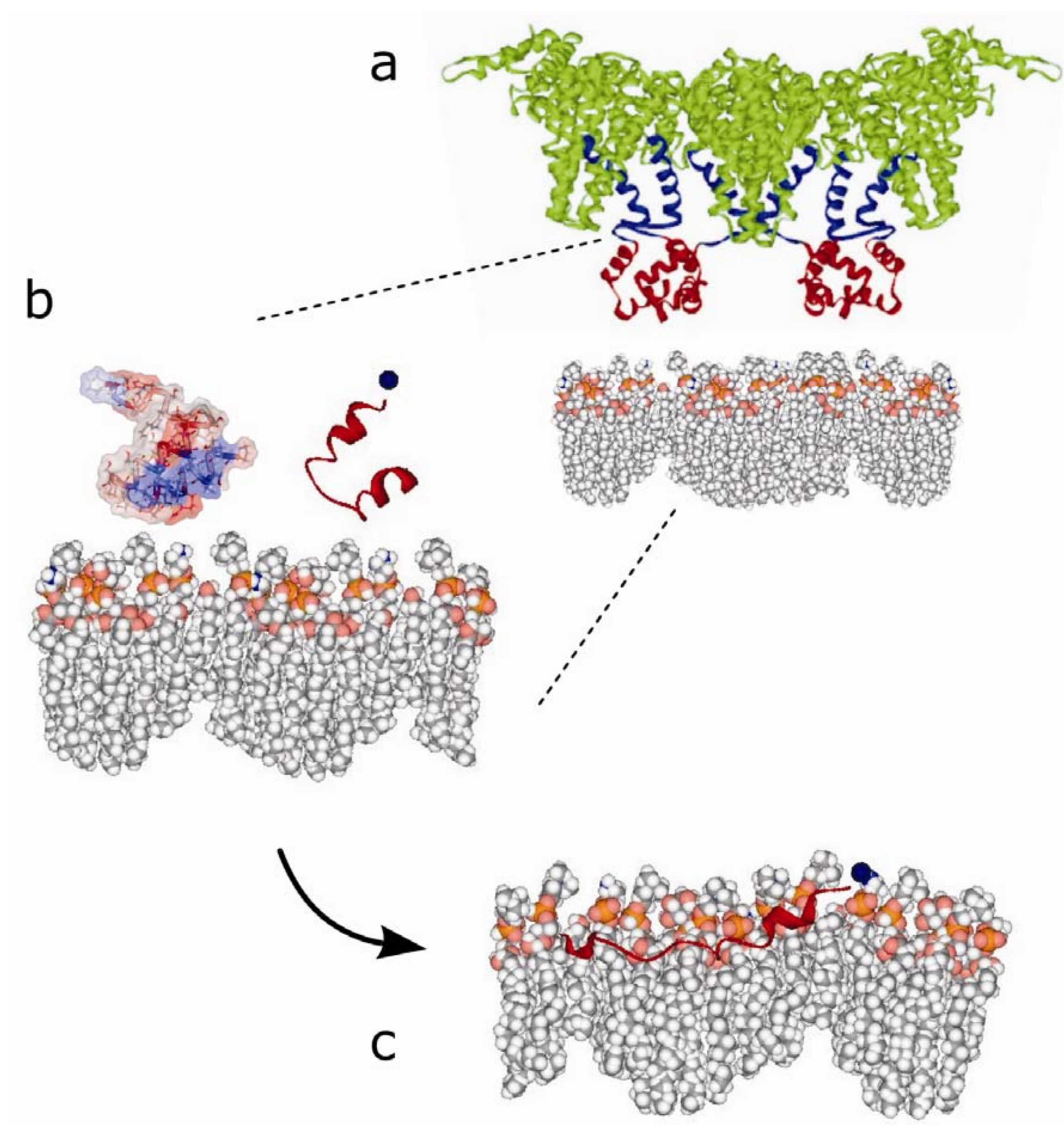

Figure 44: Prediction for double TLM capsid interaction with the outer cell membrane

According to Figure 29 the double TLM exposed on the surface of the capsid (**a**) is able to interact with the outer membrane of a target cell (a part of HBV capsid in green). (**b**) First the outer, hydrophilic parts of the double TLM hairpin structure interact with the membrane (The circle at the end of the second TLM α-helix gives the position of the fused capsid). (**c**) Second the hairpin-structure folded up inside the membrane. The hydrophobic parts (blue) of the double TLM are able to interact with fatty acids inside the membrane.

The whole TLM capsid is then surrounded by the membrane. This leads to an inverted micelle (**Figure 45**). The structure labeled by a white arrow in **Figure 36** demonstrates a capsid in the moment of passing the membrane. It can be interpreted as an inverted micelle.

The equilibrium between extracellular and intracellular localization of TLM-fusion proteins can be shifted towards the intracellular localization of TLM-molecules, if the internalized molecules are trapped by intracellular binding

partners. In case of the TLM-nucleocapsids an interference with actin and tubulin filaments was observed that seems to be involved in guidance of the incoming particles towards the nucleus [216].

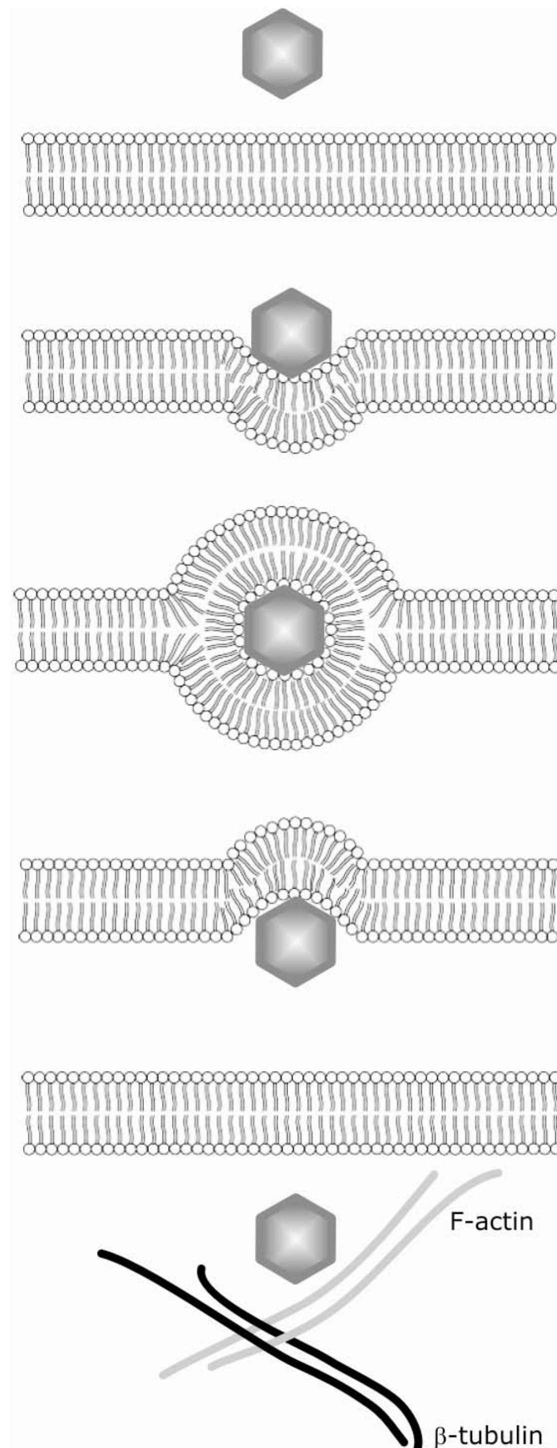

Figure 45: Model of TLM capsid translocation via an inverted micelle

The inverted micelle model for CPP translocation [211] suggests that the positively charged and hydrophilic part of the TLM on the surface of HBV derived capsids interacts with the phospholipids in the membrane, followed by the interaction of the hydrophobic part of the peptide with the membrane, creating the inverted micelle [42].

It is an interesting aspect that TLM-nucleocapsids are able to translocate the plasma membrane but fail to pass the nuclear membrane. This might be due to the different lipid composition or due to the presence of many ribosomes on the outer site of the nuclear membrane affecting the fluidity of the membrane [217-220]. Another explanation could be the presence of a yet unknown interaction partner on the plasma membrane which is not present on the nuclear membrane.

Diverse effects and explanations according to CPP translocation were described and discussed in the literature [41,209,221-223]. Known CPPs (e.g. Table 1) differ in their structures: basic for instance by being rich in arginine residues, amphipathic or cationic. Different peptides could use different pathways for translocation (e.g. by forming inverted micelles or pores in membranes). In addition, the mechanism of translocation could be dependent on whether it is the free CPP or the CPP connected to a cargo that is investigated [224].

Despite the fact that the actual pathways of CPP translocation have still to be elucidated, significant biological effects caused by the CPP-mediated increased uptake of bioactive molecules were observed *in vivo* and *in vitro*. The efforts made so far indicate that CPPs are a promising tool for future pharmaceutical applications.

IV.2 EFFICIENT GENE TRANSFER VIA TLM NUCLEOCAPSIDS

Gene therapy has become one of the most attractive therapeutic strategies and, if applicable, can treat both acquired and inherent genetic disorders. To be successful, however, gene therapy requires efficient and safe methods for delivery and targeting. Furthermore, the genes need to be expressed at therapeutic levels.

TLM nucleocapsids, as described in this study, provide a well characterized tool for efficient gene transfer into human hepatocellular carcinoma cells as well as into human primary hepatocytes. The TLM nucleocapsid mediated gene transfer displays efficiency similar to viral gene transfer systems. But in contrast to the viral gene transfer systems the new developed system offers several advantages: contaminants can be rigorously removed, unwanted viral sequences are never present and replication or other virus related modifications inside the host cell are not possible. TLM nucleocapsids translocate in almost all cells (>95%) and provide a gene expression on a moderate level in comparison to overexpression of genes resulting from common transfection systems. The expression rate of a marker gene is uniform in all reached cells. In contrast to known naked DNA delivery systems (chemical or physical transfection systems) which show

only spot-like overexpression of marker genes in the minority of cells [11] the TLM nucleocapsids reaches every cell and delivers an adequate amount of DNA into the nucleus. In addition the genetic material is packaged as in a viral system and is therefore protected against degradation. Neither treatment with DNase/RNase nor storage at 4°C can affect the activity of TLM nucleocapsids as a carrier for nucleic acids. They easily pass the cytosolic membrane of various cell types and it is assumed that they take advantage of the cellular nuclear import machinery. The nuclear localization sequence as a part of the full length C-terminus of HBcAg is assumed to be recognized as a nuclear import signal by specific intracellular receptor proteins which direct the nucleocapsid towards the nucleus. These features would be advantageous in overcoming some of the risks [225] and limitations of current viral technologies, avoiding for example, insufficient gene transfer into undesired cell types.

Two different protocols for packaging of nucleic acids into VLPs were developed. In both cases the resulting cell permeable TLM nucleocapsids are replication incompetent. The *in vitro* packaging system is based on bacterial expressed and purified truncated HBc dimers which are reassembled under the presence of foreign DNA. As major advantage this 'kit like' system combines high expression and simple purification with *in vitro* packaging of any nucleic acid (up to 3,5 kb). Except for the cell permeable capsid no virus derived proteins or genes are used. This study demonstrates for the first time that HBV capsids derived from *E. coli* and assembled *in vitro* can be loaded with foreign plasmid DNA. A drawback of this system is the small amount of capsids harboring DNA. In contrast to Zlotnick et al. [130] the capsid reassembly observed in this study was less efficient. Presumably the presence of plasmid DNA affects the process of reassembly. With a size of about 3.5 kb the used pVax1SHBs plasmid is equivalent to the size of the HBV genome. However the HBV genome is composed of relaxed-circular, partially double stranded DNA whereas the used plasmids are mostly supercoiled and more rigid. Similar observations were made with a polyoma VPI virus like particle expressed in *E. coli* [226]. Braun and coworkers described only a minor fraction of plasmid DNA packaged into the polyoma VLP. Another reason for less efficient packaging could be related to the truncated C-terminus of HBcAg. Le Pogam recently described the importance of HBcAg amino acids 165-173 for packaging and encapsidation of viral nucleic acid [227]. When arginines and serines were systematically restored at the truncated C terminus, the core-associated nucleic acid gradually increased in both size and signal intensity. He also observed that the phosphorylation at serine 170 is required for optimal RNA encapsidation and a full-length positive-strand DNA phenotype. In case of the 152 amino

acids long capsid constructs described in this study no phosphorylation sites are present and only three arginines can build up a positively charged environment to bind and package DNA inside of the capsid [118]. Nevertheless, these experiments proof the principle that DNA can be packaged into reassembled HBV capsids *in vitro*.
The second developed protocol circumvents the described drawbacks by direct packaging of nucleic acids during production of capsids in insect cells. According to the life cycle of HBV the transcript, harboring encapsidation signals and a marker gene, was recognized by the HBV polymerase and this complex was packaged into the capsids. This protocol combines the production of recombinant capsids with the packaging of foreign DNA in a single step. Furthermore, the baculovirus expression system allows the production of full length HBcAg including the C-terminus. The size of nucleic acids that can be packaged into HBV nucleocapsids is sterically limited to about 3.5 kb. In principle, packaging depends only on the presence of HBV polymerase in *trans* and on the presence of the epsilon motive in *cis*. The epsilon motive is very small (about 60 nucleotides). No virus-specific sequences limit the size of the cargo nucleic acids. In addition, the requirement of only very small virus-specific sequences ensures the high biological safety by excluding the risk of revertant virus formation [228]. Investigations using real time PCR showed that the production yield of nucleocapsids harboring DNA was significantly higher in the *in vivo* packaging system based on insect cell in comparison to the *E. coli* based *in vitro* packaging system. Nevertheless, the ratio between empty and loaded capsids produced by triple baculovirus infected insect cells was lower than expected. Presumably this low yield results on the one hand from the inhibition of reverse transcription or DNA-dependent DNA polymerase activity due to the change in the secondary structure of packaged pregenomic RNA by the insertion of a foreign gene. On the other hand the lack of regulatory cis-elements as a result of inserted foreign genes into viral genome could lead to a decreased packaging. To solve this problem, the conditions for production should be optimized to achieve higher amounts of produced nucleocapsids.
However, the amount of TLM nucleocapsids derived from insect cells was large enough to use them in a concentration of 20 nM for successful gene transfer in Huh7 cells and primary human hepatocytes. In contrast to the small number of PHHs expressing foreign genes after treatment with recombinant HBV or HIV described in previous reports [191,229], in the experiments shown in this study almost all PHHs expressed GFP or HBs marker genes. Furthermore, no apoptotic or morphological changes were observed in PHHs after treatment with TLM nucleocapsids. In regard to

biological safety and gene transfer efficiency the TLM nucleocapsids show a potential to outclass other gene delivery systems (e.g. infective virus derived systems).

IV.2.1 Cell and tissue specificity

One of the major drawbacks associated with using CPPs is their unspecific nature. CPPs have been shown to translocate a vast range of cell types *in vitro* and are distributed in several organs *in vivo*. To have potential as an important pharmaceutical tool, the translocation of CPP must be capable of cell specificity. Because the mechanism by which CPP enters the cell has yet to be elucidated, little is known about the cell specificity of CPPs. Recently, a synovial fibroblast-specific protein transduction domain (PTD) was reported to induce explicit delivery of apoptotic agents and thereby induces specific cell death [230].
Considering *in vivo* applications this can be achieved by tissue-specific promoters or by generation of nucleocapsids that are modified to allow cell selectivity. The TLM nucleocapsids described in this study display no tissue specificity. Therefore, the tissue-specificity was achieved by using liver-specific promoters (HBs promoter) upstream of the reporter genes. Combing interaction of a cell specific surface molecule with a TLM un-masking mechanism makes such selectivity possible. First of all interaction with a tissue specific surface molecule directs the particle to the surface of the target cell. Next the TLMs are unmasked which enables the entry into the target cell. Without affecting the capacity to assemble, capsids can be easily modified [110]. The previously reported hepatocytes-specific preS1 region insertion into the spike tip of HBV capsids [231] can be used to direct the TLM nucleocapsids to the surface of liver cells.
Repetitive application of TLM-nucleocapsids *in vivo* might result in an activation of an immune response induced by the immune dominant epitope localized in the spike tip of the core particle. However, as demonstrated by Nassal and coworkers [67] this epitope can be easily destroyed by insertion of foreign sequences without affecting the capacity to form properly assembled particles.
Otherwise the activation of an immune response could be of advantage. HBV nucleocapsid represents one of the best and well-studied protein-engineering carriers for insertion and exposure of foreign epitopes [232]. Recombinant molecules on the basis of cell permeable TLM capsids and packaged with DNA coding for other epitopes are suitable for *in/ex vivo* vaccination [233]. Cell permeable capsids harboring foreign epitopes in the spike tip should allow a very efficient cytoplasmic processing resulting in

MHC class I-dependent presentation of the epitope. After dissociation of the cell permeable capsid and delivery of DNA into the nucleus, the host immune system could responds directly against the epitopes exposed on the vector particles and against the expressed product of gene transfer. In contrast to conventional vaccines, a combination of protein and DNA vaccination leads to induction both humoral and cellular immune responses. Also a genetically manipulating of stem cells *ex vivo* could be possible [234].

IV.3 HBV BIOLOGY

Little is known about the intracellular way of nucleocapsids after its escape from the endosome towards the nucleus during the early steps of HBV infection [21,235]. This is caused by the fact that there is no efficient *in vitro* infection model for HBV. Our cell permeable TLM-nucleocapsids could serve as a novel tool to improve the knowledge of intracellular trafficking of HBV nucleocapsids. Pioneer work in this field was done by Kann and coworkers [94]. However most of these experiments were performed based on digitonin-permeabilized cells.

Application of cell permeable nucleocapsids allows the transfer of nucleocapsids right into the cytoplasm without affecting the integrity of the cell. It is possible to subsequent analyze the intracellular trafficking in living, intact hepatoma cells. Analyzing the data of translocation - in particular the kinetics (**Figure 40**) - a noticeable colocalization of fully assembled capsids with the cytoskeleton during the traffic was observed [216]. 60 minutes after TLM nucleocapsids translocate into hepatoma cells significant change of F-actin staining was observed. As recently reported for HIV [236] the incoming HBV capsid could stimulate the actin filament network reorganization. Passive cytosolic trafficking of virus-sized particles is generally restricted by the properties of the cytoplasm, and structures like viral capsids are unlikely to move within the cells by diffusion alone, suggesting that their efficient intracellular transport requires interactions with the host transport system [237,238]. In this study the appraised velocity of transverse HBV nucleocapsid transport was about 0,15 µm/min. This eliminates the possibility of free diffusion inside the cytoplasm. For other viruses retrograde transport velocities was measured for instance between 50 µm/min (Vaccinia virus, diameter of core: ~250 nm) and 10 µm/min (Adenovirus, diameter of core: ~90nm) [239,240].

The rim like distribution of TLM nucleocapsids in the perinuclear region was unequal and presumably the capsids interact with the microtubule organizer center (MTOC). It was observed that this interaction was stable during cell

division at the different stages of mitosis. Even after the nuclear membrane was dissolved the nucleocapsids do not enter the former nucleus (data not shown). Results show that HBV nucleocapsids can directly interact with β-Tubulin. In most cases, the minus ends of microtubules (MTs) are anchored at the perinuclear MTOC. Viral capsids (e.g. adenovirus [241], herpes simplex virus [173], respiratory syncytial virus [242], HIV [243] and vaccinia virus [239]) have been observed in close association with MTs during infection. In the case of herpes simplex virus type 1 and adenovirus type 2, the associations with MTs shortly after infection of fibroblastic cells are most probably functional. This was investigated by pharmacological depolymerization of the MTs which abolishes intracellular virus transport but does not affect virus entry into the cytosol [173,241].

HBV must deliver their genomes to the nucleus of the target cell to initiate an infection. Kann and coworkers described that during the maturation of capsids phosphorylation results in the exposure of the C-terminal sequences including NLS on the capsid surface. By this nuclear import the capsid uses the classical pathway of a surface-exposed NLS and interactions with the nuclear transport receptor Impβ via the adapter protein Impα [94]. According to the work of Seifer et al. [244] in this study the maturation of HBV nucleocapsids takes place in triple infected insect cells. The specific encapsidation of the nucleic acid is, however, mediated by HBV polymerase [192]. The exposure of C-terminal sequences is thus coupled to the presence of the viral polymerase and to genome maturation [169]. After incubation of Huh7 cells with maturated TLM nucleocapsids the fully assembled particles translocate into the cytoplasm. Thenceforward this reflects the early post entry steps of HBV infection. The active transport of TLM nucleocapsids ends at the nuclear membrane and NPC respectively. As shown by long term observations of Huh7 cells (up to 48 h) nucleocapsids were not found within the nucleus. This was confirmed by immunofluorescence and electron microscopy. In addition to that, after incubation of isolated intact Huh7 nuclei with wildtype or TLM nucleocapsids no capsid signal was detected inside the nuclei. All capsids were bound at the nuclear membrane and show a rim like fluorescence signal. Does this mean that the capsids are unable to enter the nucleus? It is not possible for nucleocapsids to translocate through the nuclear membrane and it seems that they are unable to pass the NPC of isolated nuclei as complete assembled particles. But using the new model it was observed, that the DNA packaged in the TLM nucleocapsids was efficiently expressed in Huh7 cells or PHH. Therefore the nucleocapsids transport the DNA to the nucleus, they have to partially disassemble and guide the DNA into the nucleus for gene expression.

In another set of experiments digitonin permeabilized Huh7 cells were used. According to the protocol of Rabe et al. [169], after washing out the cytoplasm the residual nuclei were incubated with nucleocapsids in the presence of ATP and rabbit reticulocyte lysate (as the source of cytosolic proteins). After 120 minutes a nuclear staining was indeed observed. By using the conformation specific MAb3120 which exclusively recognize fully assembled HBV capsids is was demonstrated for the first time that only dimers or oligomers can enter the nucleus. This suggests that the final transport of nucleic acid into the nucleus does not require the fully assembled nucleocapsid. These results were contrary to the findings of Kann and coworkers [169]. He described by using a polyclonal anti HBcAg antibody the accumulation of assembled capsid proteins inside the nucleus. But it can not be excluded that a high concentration of core dimers inside the nucleus leads to a reassembly of viral capsids.

It can propose that the new model presented in this study indeed seems to describe the post-entry intracellular transport of the nucleocapsid. Taken together, these results suggest that following entry into the cell and escape from the endosome, HBV capsids traffic along the actin/microtubule cytoskeleton towards the nucleus by passing the MTOC in a directed and active transport. HBV capsids must leave these structures and establish an association with the nuclear envelope. In a final step fully assembled cores bind to the nuclear pore complex (NPC) at the nuclear membrane and disassemble in front or inside of the NPC [169]. This finally resulted in the expression of the viral genome. These results show that cell permeable HBV nucleocapsids are able to serve as a potent tool to investigate the early steps of HBV infection.

IV.4 OUTLOOK

The ability to deliver large molecules is a considerable challenge because of the restriction imposed by the cell membrane. As rapid advances in cell and molecular biology lead to a proliferation of potent molecules that cannot be effectively delivered to cells and tissues by conventional means, a continuing refinement of the new delivery methods will be essential to realize the potential of these molecular drugs. The use of CPPs for cargo delivery represents a novel and promising approach. The TLM is a valuable tool for the delivery of high molecular weight and particulate agents into a wide range of cells. TLM has low molecular weight, no toxicity, and can be universally employed.

To further improve the biological safety - to be independent from viral polymerase and packaging signals - the double TLM has to be fused to full

length *E. coli* derived core proteins. The *in vitro* packaging by reassembling of TLM capsids needs to be optimized. To address this optimization first the structure of nucleic acids should be changed. In according to the HBV genome relaxed circular or as an alternative shorter and linearized DNA could be used. In a second step the conditions for reassembly of capsids needs to be modulated. In the future it could be possible to mix prepared TLM capsid dimers up with the required nucleic acid to easily generate a gene transfer system for a broad range of applications. Furthermore this 'kit like' system can also be used for delivery of RNAi or drugs. It could help to enhance the half-life and solubility of drugs and to decrease their toxicity. The packaged nucleic acid or bioactive molecules will be protected and delivered *in vivo* into almost all cells for instance in non-dividing cell types. E.g. it can serve as a vehicle for apoptosis inducing drugs by direct injection into a tumor.

The success of the TLM-mediated strategy for clinical use will be depending not only on their efficiency and safety but also on the ultimate cost. Large-scale applications and new methodologies are being implemented to increase the yield and reduce the cost.

The screening of new HBV drugs may be conducted by a more specific, simple and safe method through this system in the future. Also HBV infection can be investigated in a more specific manner. More interaction partners of the HBV capsid could be detected under more physiological - real infection like - conditions. With this new system and the set of antibodies described in this study it would be possible to monitor all post entry HBV infection steps. It would be possible to answer the question of capsid conformation during the first entry and re-entry of nucleus when infection occurs.

Finally the innovation of cell permeable TLM capsids could be transferred to other viral capsids for instance those with greater size. The TLM technology could help to investigate other intracellular viral pathways.

The results shown in this study indicate that cell permeable HBV-derived nucleocapsids provide a novel tool for safe and efficient gene transfer as well as for the investigation of HBV biology.