III RESULTS

III.1 BACTERIA DERIVED NUCLEOCAPSIDS

III.1.1 Generation of cell permeable core particles

To obtain cell permeable core particles, bacterial expression constructs encoding fusion proteins of the TLM and HBcAg protein were generated. Functionality of the TLM requires its exposure on the surface of the mature core particle. The C-terminus of hepatitis B core protein is basically located inside of the fully assembled capsid [118,174]. Therefore an N-terminal fusion or insertion into the ORF of HBc was recommended. Modeling analyses of the HBV core particle assembled from HBcAg monomers modified with a TLM were done according to the electron microscopy- and x-ray-based structural reconstruction [111] and extensive mutagenesis analyses of HBV core [119,175] to analyze possible exposure of TLM at various insertion positions. Two major modification sites for generation of expression constructs were chosen, the spike tip of HBcAg and the N-terminus. The TLM was inserted into the spike tip between amino acids 79 and 80 (**Figure 14**). In assembled capsids the N-termini of each monomer locates close to the surface of the particles [176]. To ensure the exposition of the TLM on the core surface in case of the N-terminal fusion a 20 aa encompassing linker derived from the HBV precore domain was inserted between the TLM and the HBcAg.

To enable efficient enrichment by affinity chromatography the coding sequence for a Strep-tag was included in all constructs between the TLM and the linker. The arginine rich C-terminal region of HBc (aa 150-183) has a uncommon codon usage in *E. coli*. To circumvent this problem, C-terminally truncated core protein constructs (aa 1-149/152) were used to ensure a high production level in bacteria. The 152 aa construct has at least three Arginine residues located at the inner surface of the capsid. Ionic interaction between these positively charged amino acids and the negatively charged DNA might facilitate DNA packaging. The constructs used in this study are based on HBV ayw subtype, and subcloned into the pASK7 bacterial expression vector.

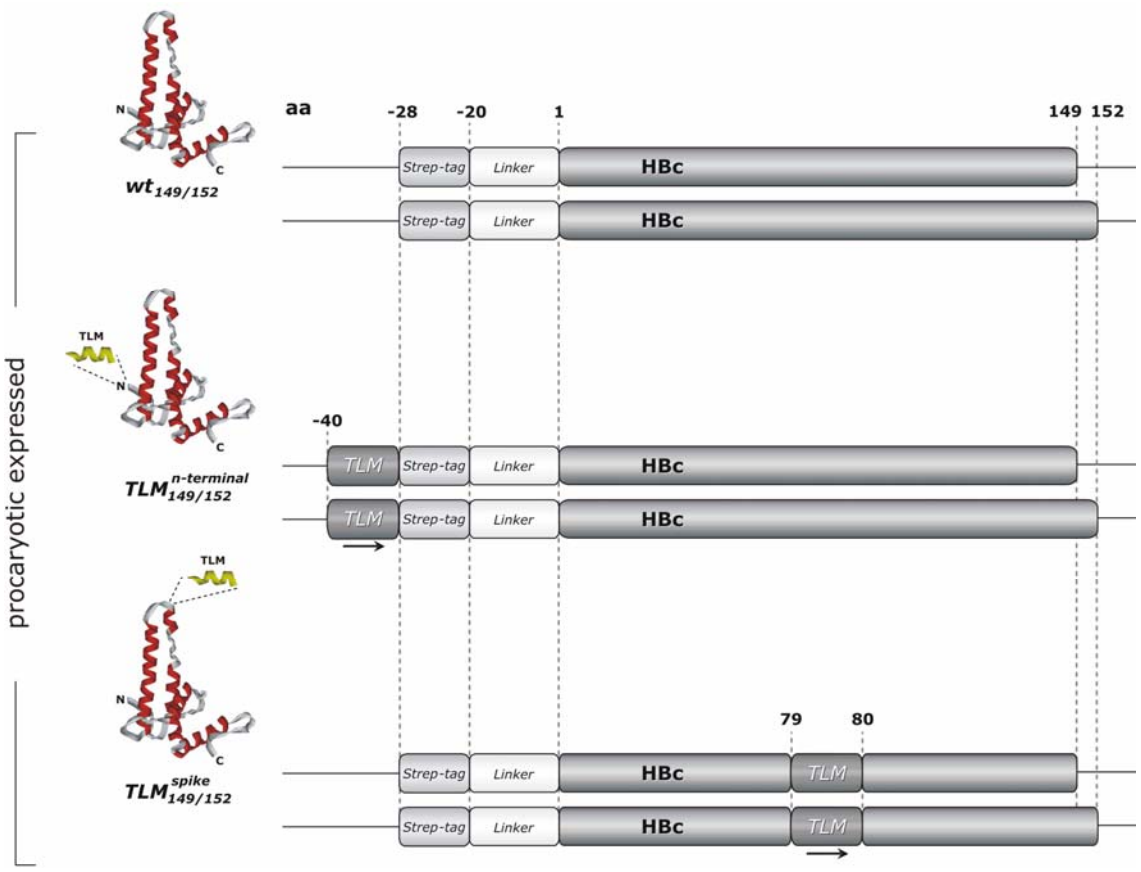


Figure 14: Structural features of wildtype and TLM-capsid constructs.
149 and 152 aa long wildtype (wt) and TLM-capsid constructs are based on the HBV ayw subtype and were subcloned into the pASK7 (IBA) bacterial expression vector. All constructs containing a 20 aa long linker peptide derived from the HBV precore and an 8 aa Strep-tag for affinity purification. The 12 aa TLM motif was fused to the N-terminus or inserted into the spike tip of the truncated, assembly-competent core proteins (149 aa). The last three arginines of the 152 aa core protein are the first part of the RNA/DNA binding domain.

Purified core particles were isolated from *E. coli* by a combination of affinity and size exclusion chromatography. Harvested bacteria cells were lysed under native conditions by French press and then centrifuged. The supernatant was applied onto a 1-ml Strep-tactin column. After elution fractions containing the native core protein, were determined by Western blot analysis, pooled and then concentrated. To distinguish between dimers and fully assembled capsids size exclusion chromatography on a calibrated superose 6 column was performed (**Figure 15**). Only assembled particles were used for further experiments. To ensure that TLM fusion did not affect the ability to assemble particles purified wildtype were compared with modified particles. Detailed analyses of the various TLM capsids on a calibrated gel filtration column showed no difference in migration of wt and modified capsids.

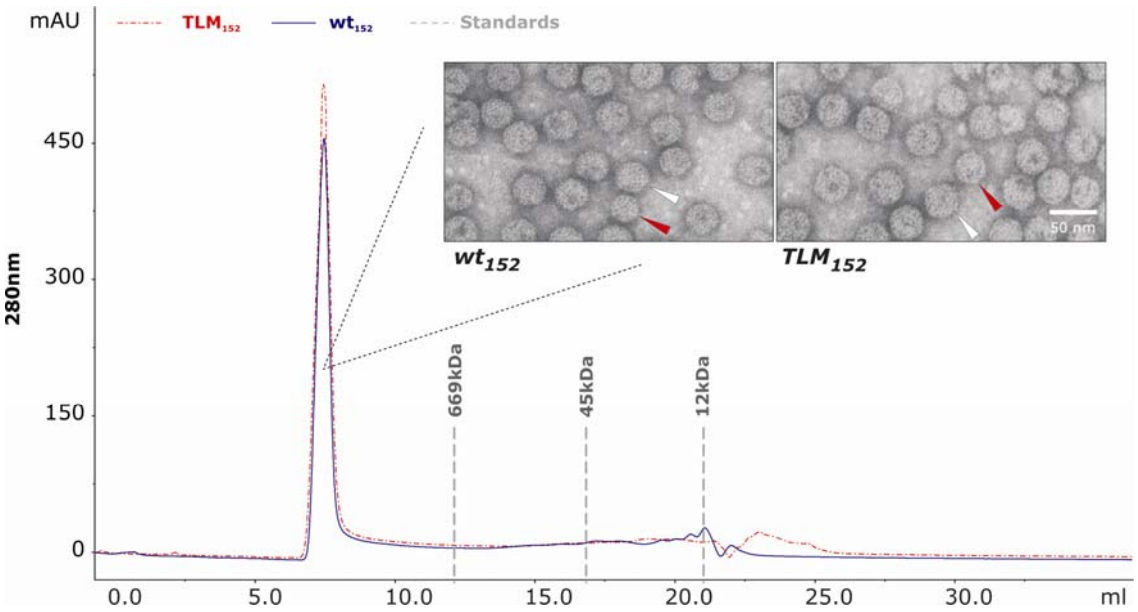


Figure 15: TLM-core fusion proteins form properly assembled core particles.
Wildtype (wt_{152}) and TLM-capsids (TLM_{152}) were bacterial expressed. These capsids were purified by affinity chromatography followed by size exclusion chromatography on a calibrated Superose 6 column (standards: Thyroglobuline [669 kDa], Ovalbumin [45 kDa] and Cytochrom C [12 kDa]). **Inlet**) Fractions of the core-peak were analyzed by transmission electron microscopy (TEM, negative stained). T3 symmetry (red arrow) and T4 (white arrow) are detectable (scale bar: 50 nm).

Furthermore, the elution profile indicates that almost all of the purified proteins are assembled to particles. This was confirmed by electron microscopy (**Figure 15**, **inlet**). The electron micrographs of various TLM core particles revealed that fusion of the TLM to HBcAg does not affect the capacity to form regular particles (
Figure 16). Surprisingly the recombinant N-terminal fused $TLM_{149/152}$ capsid expression is significant higher compared to the expression level of the wildtype capsid. This was observed by measuring the 280 nm absorption (Figure 15) and by transmission electron microscopy of purified capsids (
Figure 16). In both cases the T3 and T4 symmetries of capsids as well as the structure are in an equal ratio and the quality is identical.
Contrary the expression of the spike tip inserted $TLM_{149/152}$ construct is much weaker. Assembled capsids are indeed purified but the insertion affected quality and stability of the capsids. Irregular capsids of different size and structure filled by contrast medium are detectable.
The additional three Arginines residues at the C-terminus in case of the HBc_{152} constructs did not have any negative influence on structure, quality and expression level of the capsids but might prove important for DNA packaging. Therefore, the N-terminal modified capsids truncated at aa 153 were used for further experiments. The described bacterial expression

system provides the opportunity to easily generate recombinant hepatitis B capsids under native conditions in large amounts and purity.

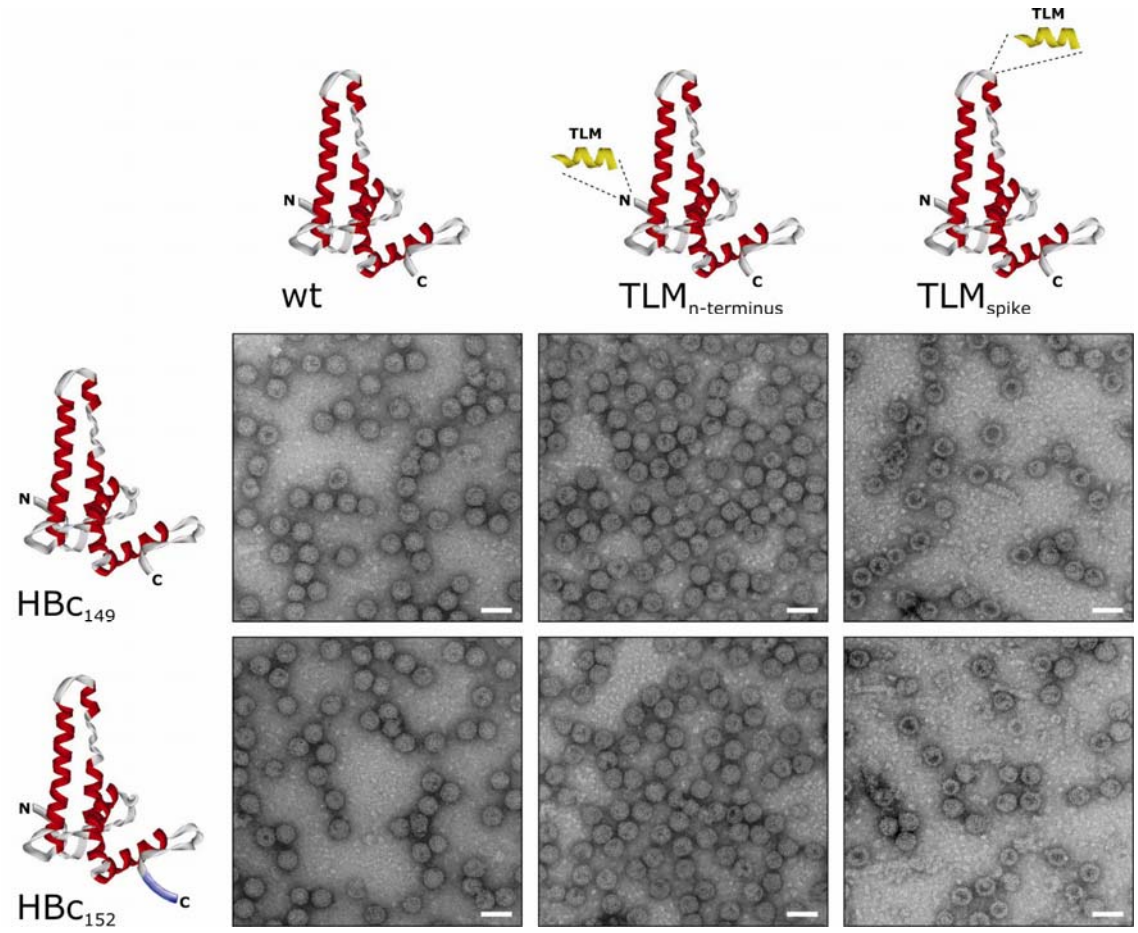


Figure 16: Effect of TLM insertion on capsid formation.
$Wt_{149/152}$ and recombinant $TLM_{149/152}$ capsids derived from the same amount of *E. coli* lysate were purified and analyzed by TEM (negative stained, scale bar: 50 nm).

III.1.2 Cell permeability of the TLM capsid

In order to develop a new gene transfer system with virus like particles as vehicles the cytoplasmic membrane as the main barrier of a cell has to be overcome. As previously shown TLM fusion proteins have the ability to translocate across the cell membrane into the cytoplasm of eukaryotic cells [52,54].

The following experiment was performed to prove the initial idea that fusion of cell permeable TLM peptides at the surface of fully assembled capsids generates cell permeable capsids. As a model system to investigate the translocation of TLM capsids, human liver cells (Huh7) were used. Huh7 cells were incubated for 60 minutes under physiological conditions with purified wt and TLM capsids (20µM). After a washing step the cells were

fixed and the localization of hepatitis B core protein was determined by immunofluorescence microscopy using an HBcAg specific antibody. The cytoplasm was stained unspecifically by Evans Blue. To confirm that the TLM capsids are inside the cell, their intracellular localization was examined in this and the following experiments by confocal laser scanning microscopy (CLSM). Therefore as a control, cells incubated only with PBS were used to calibrate the CLSM and trigger the autofluorescence of cells. The intensity of lasers, the emission filter and pinhole diaphragm setup as well as the sensitivity of photomultiplier were adjusted and were used for all probes in the same way.

As shown in **Figure 17**, only in case of the cell permeable TLM core an intracellular pattern of fluorescence in the cytoplasm was observed. It was evenly distributed within the cells giving rise to both diffuse and punctuated cytoplasmic fluorescence. No TLM capsids were detectable inside of any nucleus. There were, however, differences in the amount of internalized TLM cores. While most cells exhibited uniform protein fluorescence of varying intensity, 15–25 % of the cells shown no specific staining at all. In contrast to the TLM modified capsid the wildtype capsid was not able to enter the cells in any case.

When cells were incubated with capsids and fixed without a washing step membrane associated wildtype and TLM capsids were detectable (data not shown). After an intensive washing step it was easy to distinguish between unspecific membrane association and translocation of the capsids into the cytoplasm of cells.

These results demonstrate that TLM peptides exposed on the surface of hepatitis B capsids can mediate cell permeability of complete particles. The intention of the reported results is to present a proof of principle that the cell permeable capsids can be instrumentalized as a shuttle for gene transfer. To increase the amount of internalized cells as well as the level of translocated capsids the TLM constructs were optimized as described later.

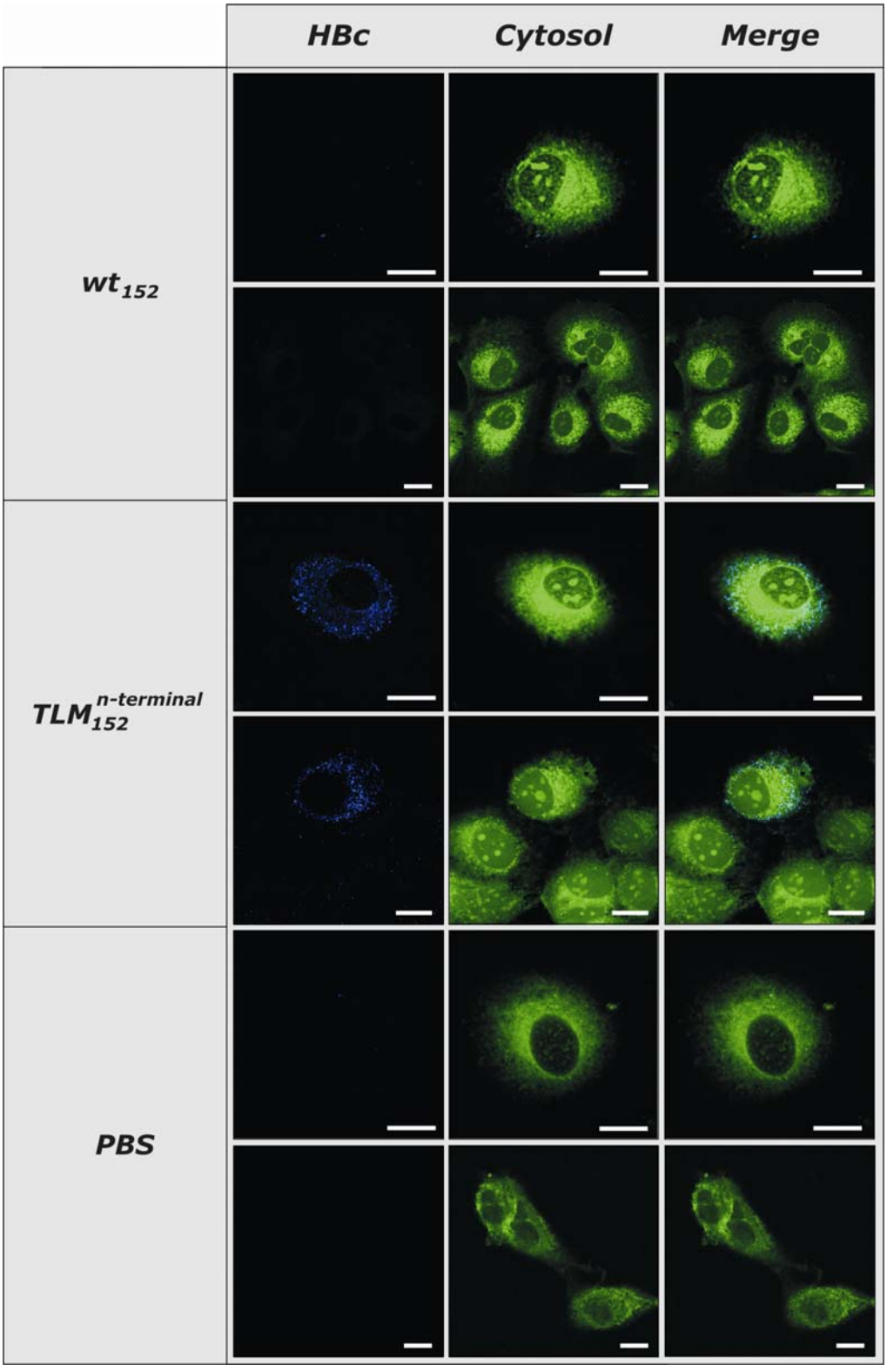


Figure 17: Fusion of the TLM to the core proteins generates cell-permeable particles.

Confocal laser scanning immunofluorescence microscopy (CLSM) of Huh7 cells grown for 60 minutes under physiological conditions in the presence of purified wildtype (wt_{152}) and N-terminal fused TLM_{152} core particles (in each case 10 nM). PBS buffer was used as negative control. For detection of cores the HBcAg specific antibody (Biomeda) was used. The cytosol was stained unspecifically by Evans Blue (scale bar: 50 µm).

III.1.3 *In vitro* packaging of nucleic acid into capsids

Gene transfer by cell permeable nucleocapsids requires (i) cell permeable capsids and (ii) efficient packaging of nucleic acid into the capsids. After having generated cell permeable capsids which serve as a shuttle, the next task for establishing a new gene transfer system was to package DNA harboring a reporter gene into the cell permeable capsids. The emerging nucleocapsids would be able to translocate into cells as well as to protect nucleic acid on the way towards the nucleus. Therefore it was necessary to develop a system for packaging foreign plasmid DNA into cell permeable capsids.

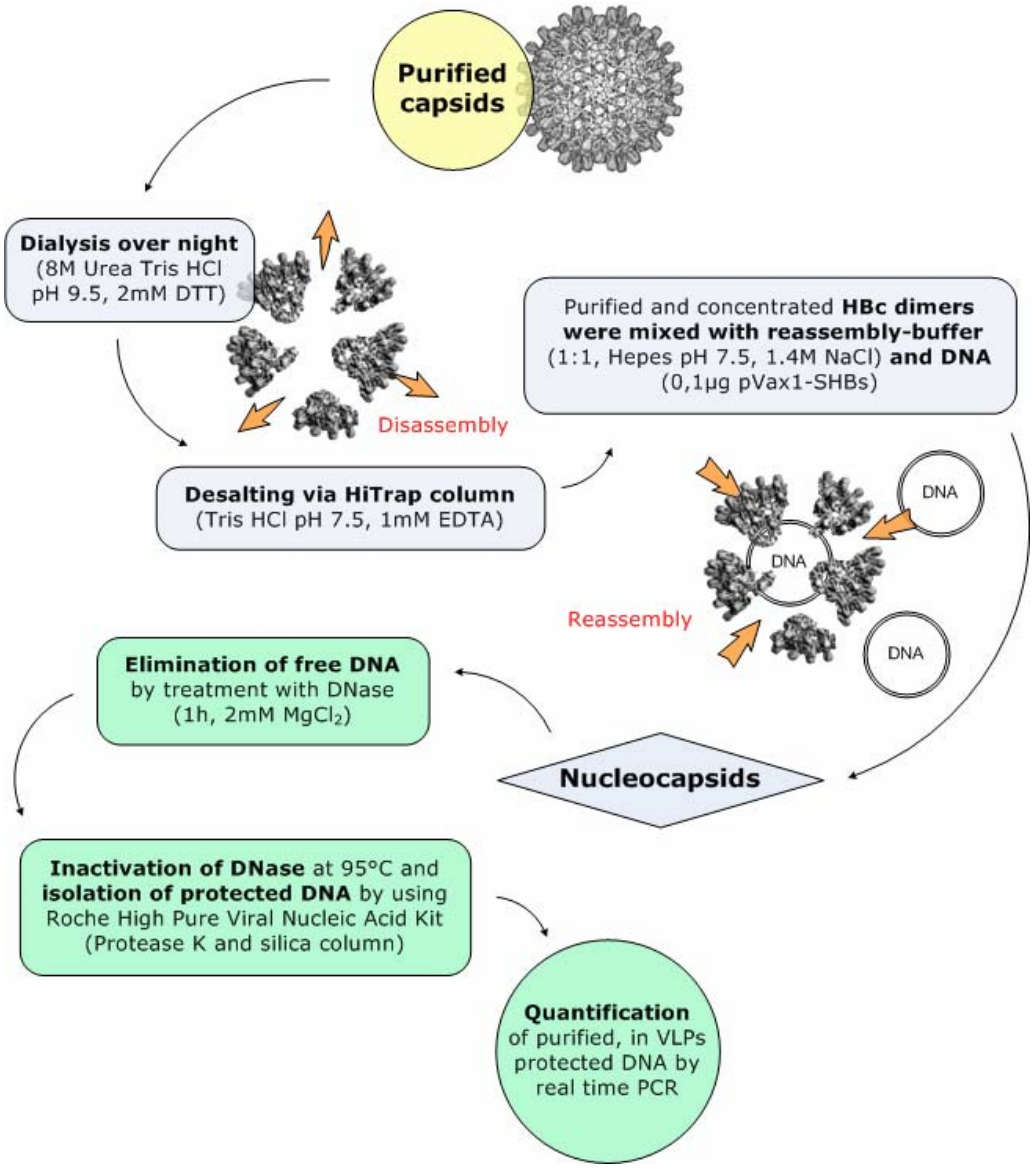


Figure 18: Schema of in vitro packaging of plasmid DNA into capsids.
Hepatitis B capsids were disassembled (dialyzed against 8M Urea) and reassembled in the presence of plasmid DNA (containing a reporter gene). After DNase treatment the amount of packaged and protected DNA was quantified by PCR.

The described native purification procedure results in generation of empty capsids. To package nucleic acids into purified assembled capsids it is essential to dissociate the particles followed by reassociation of the particles in the presence of DNA. As it is described in detail for the hepatitis B capsid by Zlotnick et al. [2,114,118,130,174,177,178], it is possible to dis- and reassemble HBV capsids. Dissociation of the capsids was achieved by denaturing conditions (urea), the removal of NaCl and reduction of disulfide bonds. **Figure 18** shows the conditions used to disassemble purified capsids into dimers.

Intact VLPs could then be reconstituted during a multistep reaction by increasing the concentration of NaCl and decreasing the concentration of the dissociating agent (Urea). The intermediate steps were monitored by size exclusion chromatography and electron microscopy of the core-peak fractions (**Figure 19**).

To investigate whether foreign DNA could be packaged into VLPs during the reassembling process, plasmid DNA was added to the dimers obtained after dissociation of the capsids. The preparation was then diluted in a reassembling buffer containing high level of NaCl. About 10 to 20 % of the dimers seemed to reassemble into VLPs. To demonstrate that plasmid DNA was packaged into capsids, reconstituted nucleocapsids were treated with DNase. The detection of DNA after DNase treatment is proof of its protection by packaging into reassembled VLPs. As shown in **Figure 20**, reassembled wt and TLM nucleocapsids are able to protect packaged pVax1SHBs plasmid DNA from digestion. Furthermore the amount of packaged DNA was determined by real time TaqMan PCR analysis (Figure 20b).

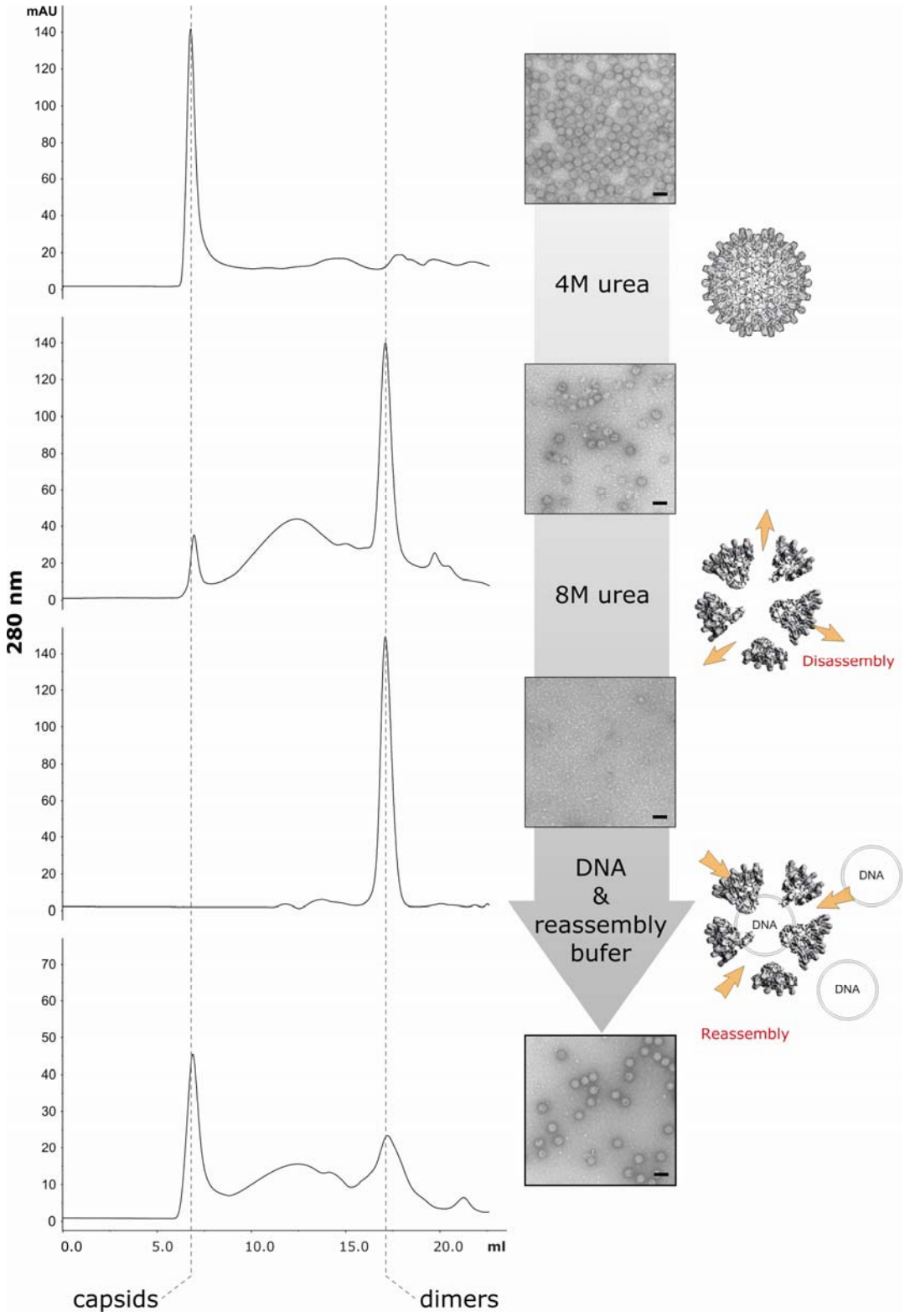


Figure 19: In vitro packaging of plasmid DNA into capsids.
Purified hepatitis B virus capsids were disassembled (dialyzed against 4M and 8M Urea) and the dimer-peak fractions were pooled and concentrated. Dialysis against reassembling buffer (containing high amounts of NaCl) in the presence of plasmid DNA (pVax1SHBs, containing SHBs as a reporter gene) leads to reassembly of concentrated dimers to capsids harboring the DNA (nucleocapsids). Consecutively the capsid-peak of superose 6 gel filtration (left hand, elution volume ~7ml) was analyzed by TEM (right hand, negative stained, scale bar: 50 nm).

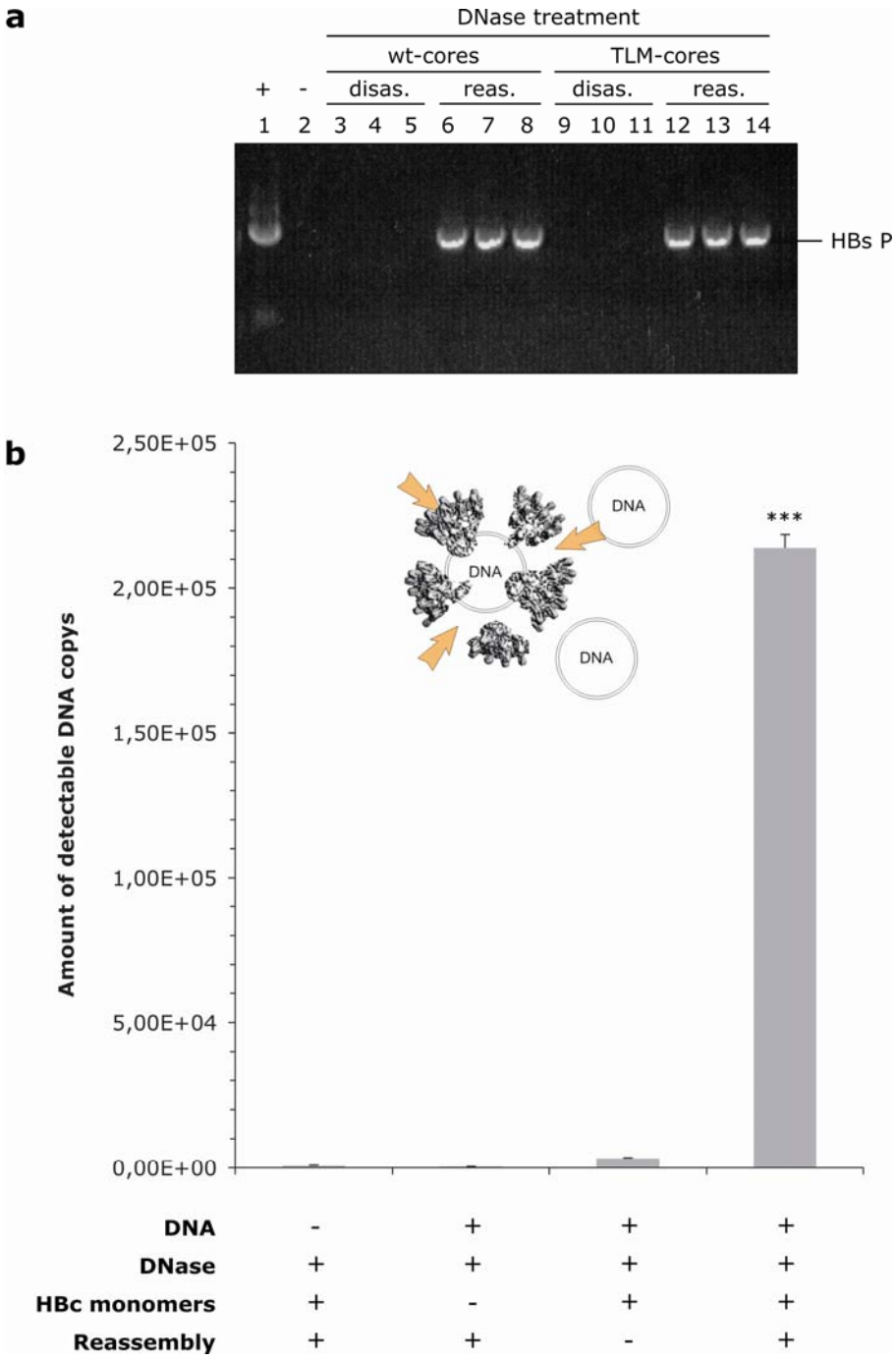


Figure 20: Quantification of packaged and protected plasmid DNA inside of reassembled cores.

a) Wildtype (wt) and TLM-cores were disassembled (disas.) and reassembled (reas.) in the presence of DNA (pVax1SHBs, containing SHBs as a reporter gene). 0,1 µg pVax1SHBs plasmid DNA and 4µg purified HBc dimers were used for reassembly. Unpackaged DNA was removed by DNase treatment (20 units, Roche). After DNase treatment a PCR using SHBs specific primer was performed (lane 1: positive and lane 2: negative control). Three independent experiments were separated on a 1 % agarose gel (wildtype cores 3-5, 6-8; TLM-cores 9-11, 12-14). **b)** Quantification of plasmid DNA packaged and protected against DNase treatment inside of reassembled capsids using TaqMan real time PCR with SHBs specific probe [179].

It was demonstrated that recombinant truncated hepatitis B viral capsids were efficiently produced in bacteria. Furthermore it was shown that under native conditions the fully assembled capsids can be easily purified in two steps. The fusion of TLM to the N-terminus of HBcAg mediates the cell permeability and this enables the capsids to translocate into Huh7 liver cells. By a newly developed protocol it was possible to disassemble and reassemble the capsids under the presence of plasmid DNA to generate nucleocapsids. The *in vitro* packaging protocol was modified in various ways (e.g. pH, ratio of DNA : HBc dimer and composition of reassembly buffer) but the major drawback of the system can be seen in the ratio between used HBc dimers and the resulting amount of reassembled capsids harboring DNA.

In comparison to many other previous described TLM applications [52,54,180] the TLM, exposed on the capsid surface leads only to an unsatisfying level of cell permeability. Furthermore the *E. coli* expression system allows only the production of truncated recombinant HBcAg proteins, missing the C-terminus and all the including advantageous functions (see Figure 7).

In order to overcome the disadvantages of unsatisfying level of cell permeability and *in vitro* packaging of bacterial expressed truncated TLM capsids, the system had to be reconsidered. A TLM fused to the core protein which is the basis of a particle with a size of more than 5000 kDa goes along with more difficulties regarding the exposition of the TLM motif on the surface of the assembled capsid then in comparison to a an other previously described cell permeable TLM fusion proteins like the TLM-HBx [54] of relatively small size (17 kDa).

Experiments with fluorescence labeled TLM peptides as well as molecular modeling with two TLM peptides as it is shown in **Figure 21** indicate a dimerization of the TLM peptide [56]. The modeling of two amphipatic TLM α-helices in the presents of water shows that a dimerization leads to an energy-minimized structure. The dimer is possible in reverse and as well in inverse orientation of the TLM peptides. The minimization of energy in case of the inverse orientation is greater (-75,7 %) than in case of reverse orientation (-64,2 %). The affinity to dimerize could be a requirement for high efficient cell permeability of described small TLM fusion proteins.

C- or N-terminal TLM peptides as part of a cell permeable fusion protein [54] are able to built dimers in front of the outer cellular membrane. Single TLM peptides in the present constructs are fixed on the surface of the huge HBV capsid and are unable to interact with each other to dimerize.

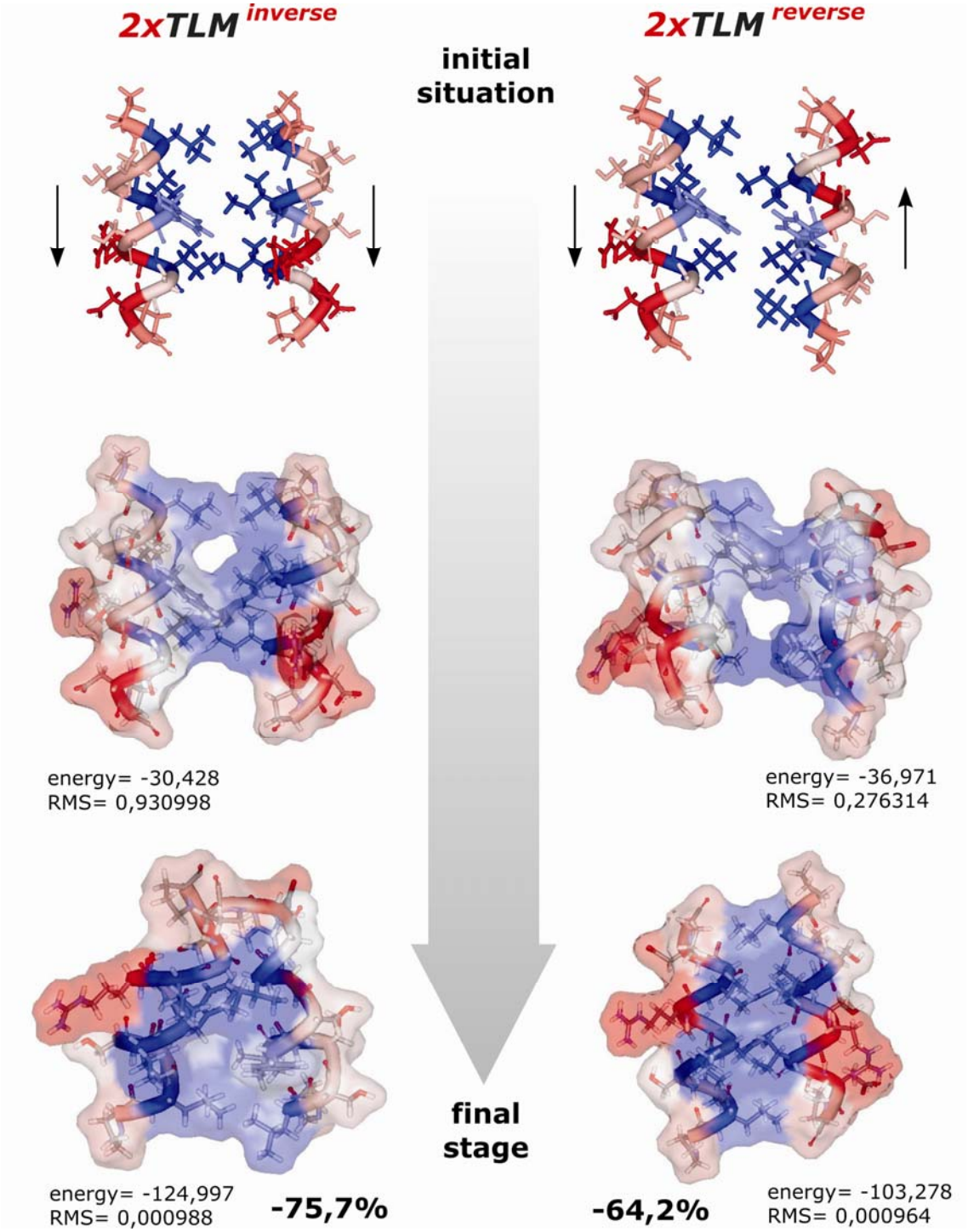


Figure 21: Analyzing the dimerization of two TLM peptides by molecular modeling. To analyze the dimerization the initial situation for modeling was the inverse and reverse orientation of two TLM peptides. Hydrophilic amino acids which form the α-helical TLM are colored in red and hydrophobic aa in blue. During molecular modeling in the presence of water the peptides reach more favorable conditions and finally end in an energy optimized stage with small root mean square (RMS) deviation (kcal/mol $\times$ Å, calculated by Polak-Ribiere algorithm) .

III.2 INSECT CELL DERIVED NUCLEOCAPSIDS

III.2.1 Generation of double TLM capsids

Computer reconstruction of dimers consisting of two TLM peptides in both possible orientations led to generation of new constructs (**Figure 22**). In addition to the new inverse and reverse double TLM fused to the N-terminus of HBcAg an untruncated core protein was used. The full-length open reading frame of HBcAg possesses of all features of the arginine rich C-terminus [181]. The HBV DNA sequence is adapted to human or at least eukaryotic codon usage. To provide an adequate expression of full-length core protein (183 aa) as well as post translation modification it was required to change the expression system.

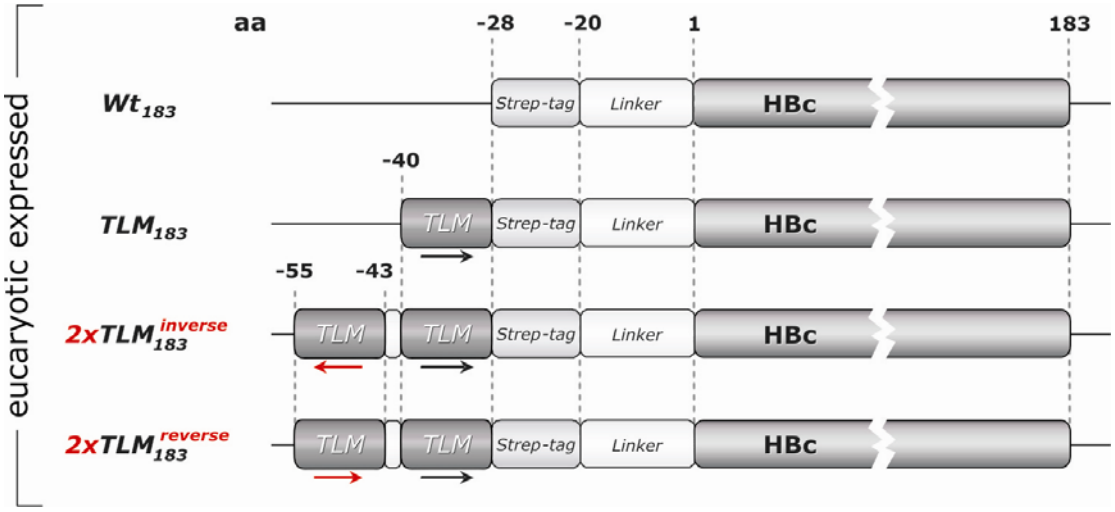


Figure 22: Structural features of full-length wildtype and TLM-capsid constructs. Full length wildtype (wt) capsid constructs (183 aa) based on the HBV ayw subtype featured a 20 aa long linker peptide derived from the HBV precore and an 8 aa Strep-tag for affinity purification. The 12 aa TLM motif was N-terminal fused to the wt. In case of the double TLM constructs the second TLM was inverse or reverse N-terminal fused. The double TLM peptides were coupled via a Serine-Glycine-Serine (SGS) linker for free rotatability of the second TLM. All constructs were cloned into eukaryotic expression vector (pCDNA3.1) and into the Baculovirus transfer vector pBacPak9. All constructs were expressed by baculovirus-infected Sf9 cells which were cultivated in stirred suspension culture.

The baculovirus expression system, which uses the baculovirus *Autographica californica* to infect *Spodoptera frugiperda* (Sf9) insect cells, offers distinct advantages over prokaryotic overexpression of foreign proteins, including: (i) eukaryotic post-translational modification of expressed protein, (ii) increased solubility of recombinant fusion proteins synthesized in insect cells leading to increased yield under mild conditions, and (iii) baculovirus-infected cells produce extremely high levels of large

and/or complex polypeptides which might be difficult to purify from prokaryotic cells. The PCR primers used for generation of recombinant full length core protein were optimized for the *Spodoptera frugiperda* codon usage. This leads to a better expression of HBcAg in Sf9 cells mediated by the recombinant baculoviruses.
The second N-terminal TLM peptide in reverse and inverse orientation was fused by a Serine-Glycine-Serine (SGS) linker to the first TLM. Glycine is very common in turns because its residue (R) group presents little steric hindrance. It enables the second TLM to rotate freely around the first one. The polar sidechains of Serine often populate position next to the Glycine in a type I turn where they can form a hydrogen bond to the backbone NH of opposite amino acid residue.

III.2.2 Cell permeability of double TLM capsid

To asses the cell permeability mediated by double TLM constructs in comparison to WT and single TLM capsids, adherent Huh7 cells were incubated with equal amounts (20nM) of purified capsids. After an incubation of 60 min, the cells were fixed and the internalized nucleocapsids were immunostained with an HBcAg specific monoclonal antibody that exclusively recognizes properly assembled particles and fails to recognize HBcAg- monomers or oligomers (MAb3120) [182]. The cytoskeleton as an intracellular marker was visualized by fluorescence-labeled phalloidin which binds to F-actin. The specimens were analyzed by confocal laser scanning immunofluorescence microscopy with the same setting.
The results illustrated in **Figure 23** show that only the TLM_{183} capsids are able to enter the cells. In contrast, no significant intracellular fluorescence signal was detectable in cells that were exposed to the wildtype capsids or PBS buffer. The three TLM_{183} constructs differ distinctly in their ability to translocate into cells. In comparison to the double TLM_{183} constructs the distribution of single TLM_{183} capsid inside the cells is different. The single TLM_{183} capsids are colocalized with the actin filaments next to the cytoplasmic membrane (**Figure 23**, white arrow). The double TLM_{183} capsids enter almost all cells. They show a bright cytoplasmic staining by indirect immunofluorescence with considerable fluorescence localized to the perinuclear region. The inverse double TLM_{183} capsid (2xTLM) showed the greatest cell permeability. Modification of TLM_{183} capsids with a second N-terminal TLM peptide - especially in inverse orientation - leads to significant increase of cell permeability. Experiments with human HepG2 liver and HeLa kidney cell lines yield similar results (not shown).

Figure 24 offers a detailed look to adherent Huh7 cells incubated for 35 minutes with inverse double TLM_{183} capsids. All cells show a significant intracellular staining for cell permeable capsids. At this time point the intensity of fluorescence differs between large cells (weaker signal) and small cells (stronger signal).

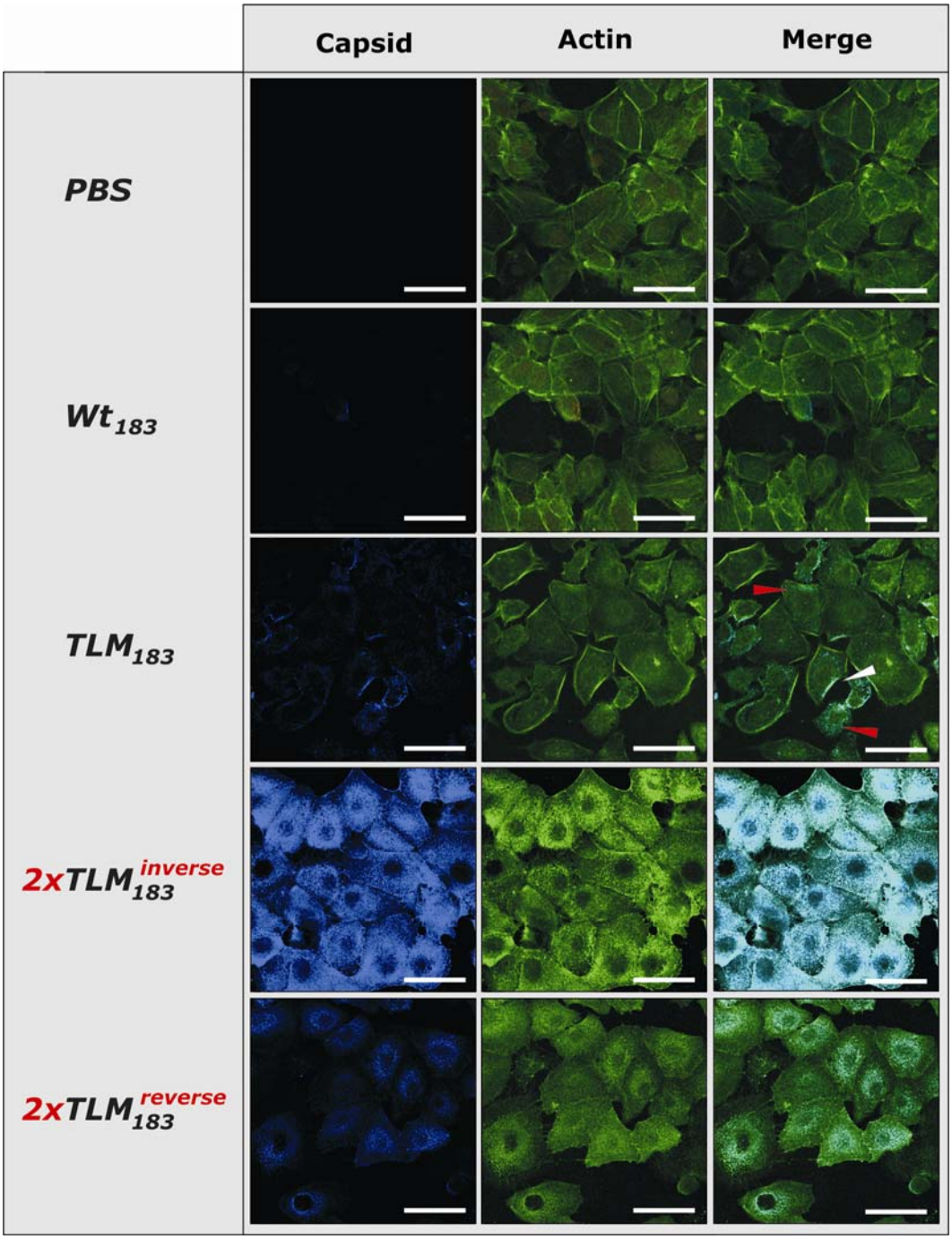


Figure 23: Cell permeability comparison of different TLM capsids.
CLSM images of Huh7 cells grown for 60 minutes in the presence of full-length wildtype (wt) as well as single and double TLM core particles (in each case 20 nM). PBS buffer was used as negative control. For detection of cores the monoclonal antibody (MAb3120) which selectively recognizes complete HBc particles was used. The actin staining clarifies the dimension of each cell (scale bar: 50 µm).

In the smaller cells an accumulation of cell permeable capsids in the perinuclear area was already detectable. In larger cells a more diffuse cytoplasmic staining was observed. The z-stack gallery shows slices from the bottom to the top of the liver cells - no nuclear staining was observed.

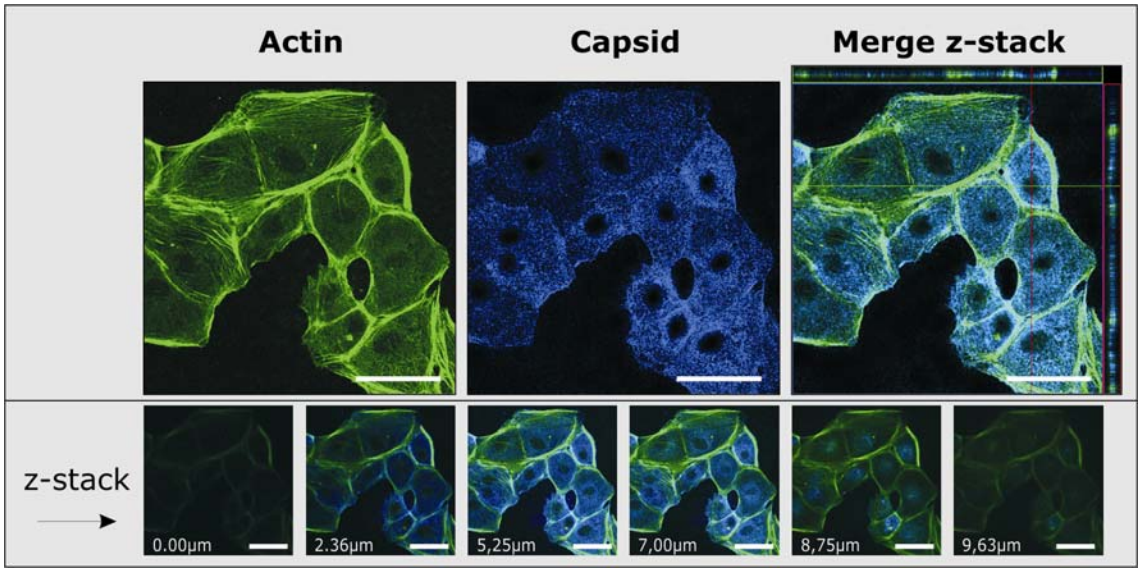


Figure 24: Detailed view of inverse double TLM capsid cell permeability.
CLSM images of Huh7 cells grown for 35 minutes in the presence of full-length inverse 2xTLM core particles (in each case 20 nM). Capsids were detected with MAb3120. The merge shows the x, y optical section through the center of the cell and x, z and y, z projections (right and upside) of this confocal image. The merge z-stack gallery shows slices from the bottom (0,00 µm) to the top (9,63 µm) of the cells (scale bar: 50 µm).

III.2.3 Time course of inverse double TLM_{183} translocation

Monitoring the time course of the cell permeable inverse $2xTLM_{183}$ capsid revealed a very rapid translocation across the plasma membrane (**Figure 25**). First intracellular signals were detectable after 5 minutes of particle incubation. After 15 minutes capsid staining was observed in almost all cells. This signal increased during the next 45 minutes and reached a maximum at around 50 minutes. A washing step followed by changing medium after 45 minutes leads to a stop of new TLM capsids penetrating the cells. Capsids diffuse located in the cytoplasm moves towards the nucleus and gave the typical perinuclear staining. After 60 minutes only a weak staining next to the cytoplasmic membrane was observed. The great majority of fully assembled inverse double TLM_{183} capsids (detected with MAb3120) were located in the perinuclear region. For all following experiments the inverse double TLM_{183} was used and often short termed as TLM_{183}.

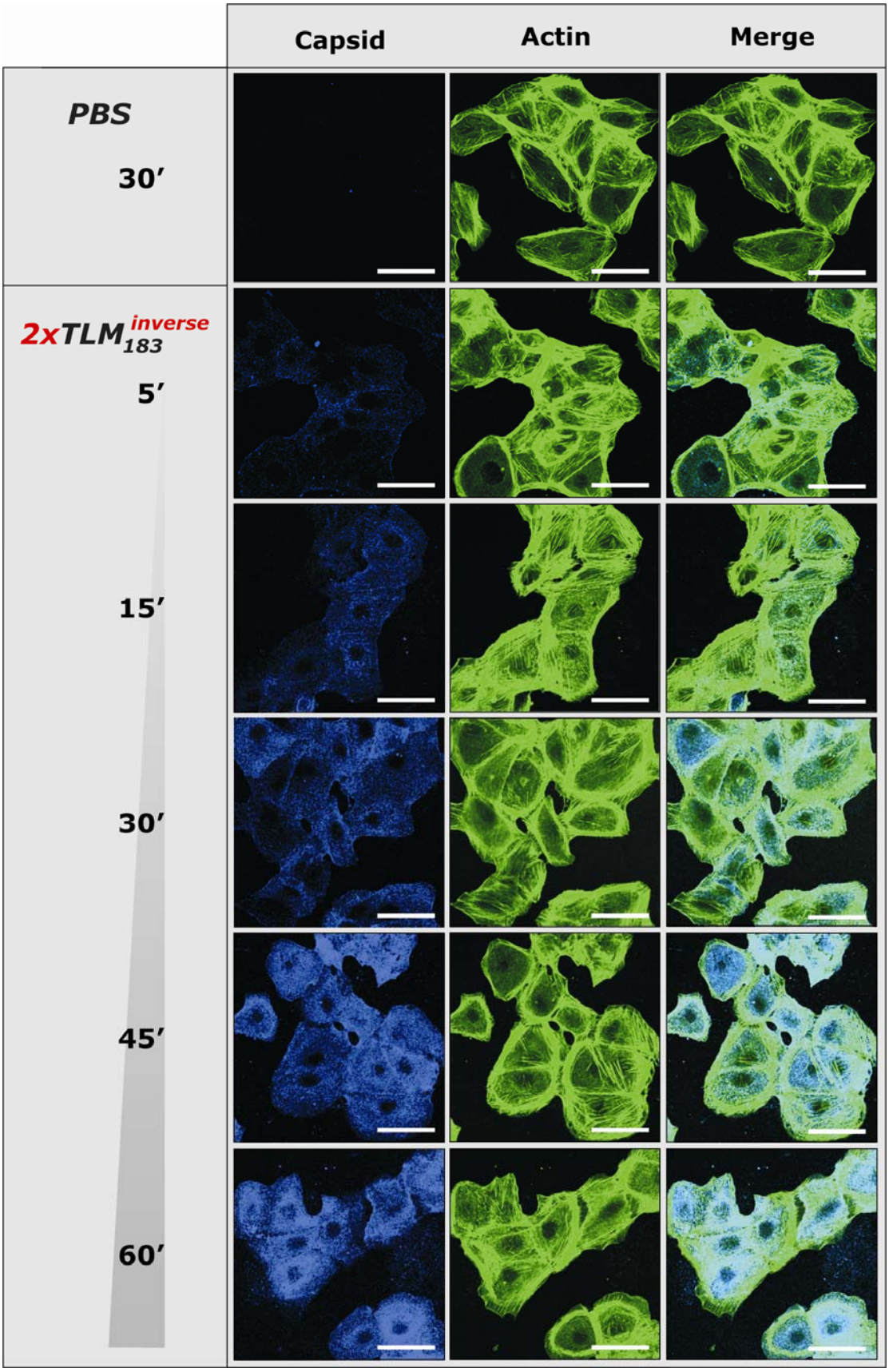


Figure 25: Kinetics of inverse double TLM capsid cell permeability.
CLSM images of Huh7 cells grown up to 60 minutes in the presence of full-length inverse 2xTLM core particles (in each case 20 nM). PBS incubation for 30 minutes serves as negative control. Capsids were detected with MAb 3120 (scale bar: 50 µm).

III.2.4 Permeability of inverse double TLM_{183} in living cells

Studies of the translocation of cell penetrating peptides (CPP) show that the observed uptake could be merely an artifact from membrane disruption when fixing cells. Disruption of cytoplasmic or endosomal membranes through harsh cell fixation results in peptides entering the cytosol and nucleus, which leads to false positive as an evidence for a translocation [28].

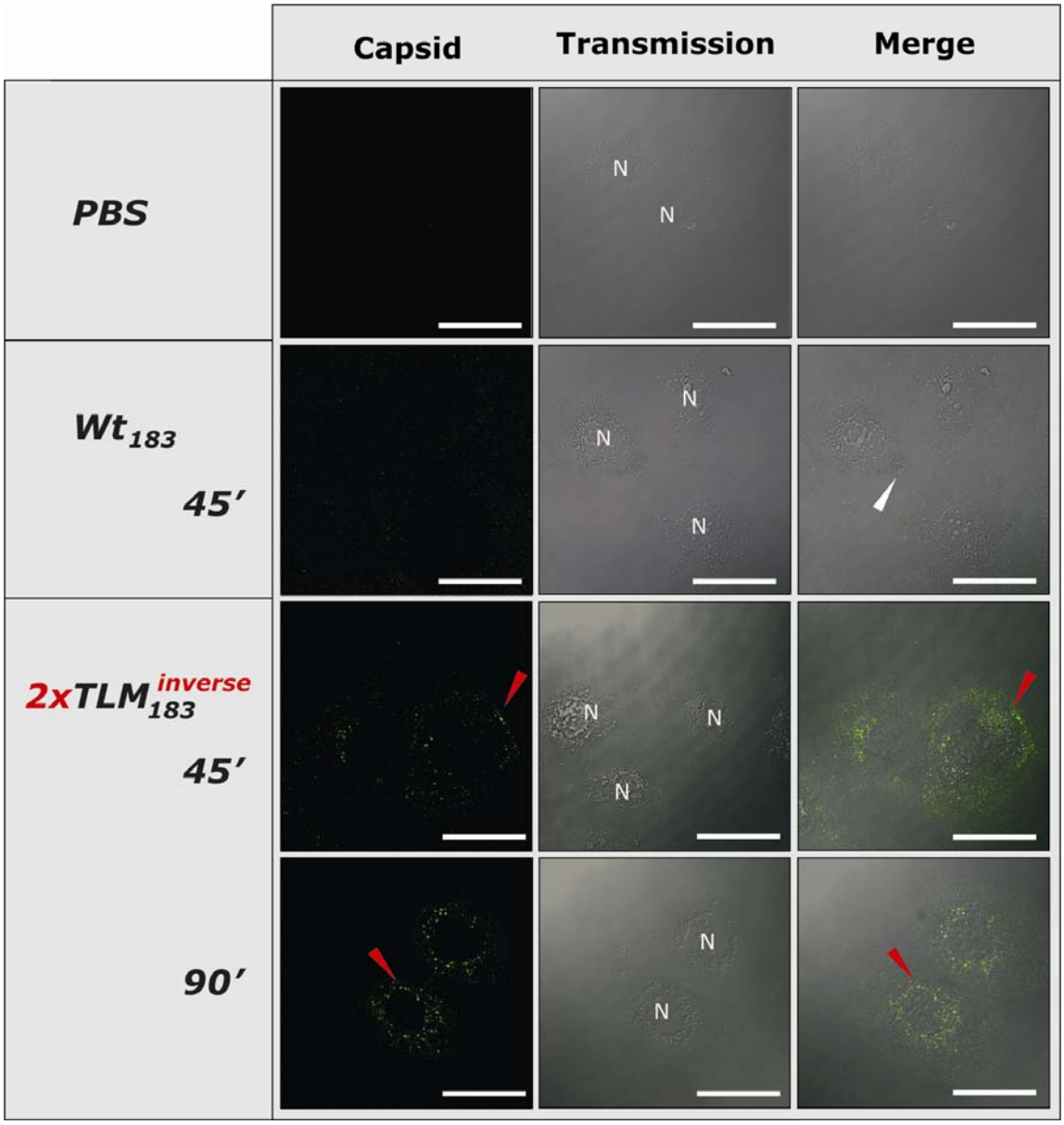


Figure 26: Life cell assay to observe the cell permeability of inverse double TLM capsids in living cells.

CLSM images of living unfixed Huh7 cells (N = nucleus) grown up to 90 minutes in the presence of FITC-labeled full-length wildtype (white arrow) and inverse 2xTLM core particles (red arrows) (in each case 10 nM). PBS incubation for 45 minutes serves as negative control (scale bar: 50 µm).

To finally exclude such artifacts (after having fixed the cells carefully) and misinterpretation of intracellular signals a real time, live cell imaging assay with fluorophore-labeled capsids was performed (**Figure 26**). As described in Experimental Procedures purified wildtype and TLM capsids were labeled with activated FITC molecules. Fluorescein was selected as a reporter group because of minimum impact on the cellular distribution of fluorescently labeled peptides when compared to other fluorescent dyes (own unpublished observations). To separate labeled capsids from free FITC molecules size exclusion chromatography was performed. Living unfixed adherent Huh7 cells were incubated for up to 90 minutes with purified FITC labeled wildtype or 2xTLM_{183} capsids.
In comparison to the fluorescence signal intensity of previously shown CLSM images the real time signals of labeled capsids are weaker. A reason for that is the labeling rate of approximately 30 FITC molecules per capsid. In contrast to that, by using fixed cells and indirect immunofluorescence the primary monoclonal antibody is able to bind more than 120 epitopes at the surface of fully assembled capsids. In addition to that the fluorescence-labeled secondary polyclonal antibody amplifies the signal by recognizing multiple epitopes on the primary antibody.
Despite the weaker signal, the FITC labeled 2xTLM_{183} capsid shows a significant cytoplasmic localization after 45 minutes (**Figure 26**, red arrows). After 90 minutes the cell permeable capsids were seen in the perinuclear region as well as directly at the nuclear membrane. A nuclear staining was not observed. In contrast, no significant intracellular fluorescence signal was seen in cells that were exposed to the wildtype capsids (white arrows) or PBS buffer.
The translocation of the FITC labeled 2xTLM_{183} capsid into the cytoplasm of unfixed cells suggests a non-endocytotic uptake.

III.2.5 Non-endocytotic uptake of cell permeable inverse double TLM_{183}

Understanding the mode of entry of TLM capsids and defining their intracellular localization are of particular interest to establish this new model system for gene delivery. To elucidate the mode of entry various metabolic or endocytosis inhibitors were used to test if the entry of cell permeable TLM capsids is energy and endocytosis independent (**Figure 27**).
Fluorescently labeled transferrin serves as positive control for endocytosis in the following experiments. Transferrin is a monomeric serum glycoprotein (MW ~80 kDa) that binds two Fe^{3+} ions to permit delivery to vertebrate cells through receptor-mediated endocytosis [183,184]. Once iron-carrying

transferrin proteins are inside endosomes, the acidic environment favors dissociation of the sequestered iron ions from the transferrin–receptor complex. Following the release of iron, apotransferrin is recycled to the plasma membrane, where it is released [185].

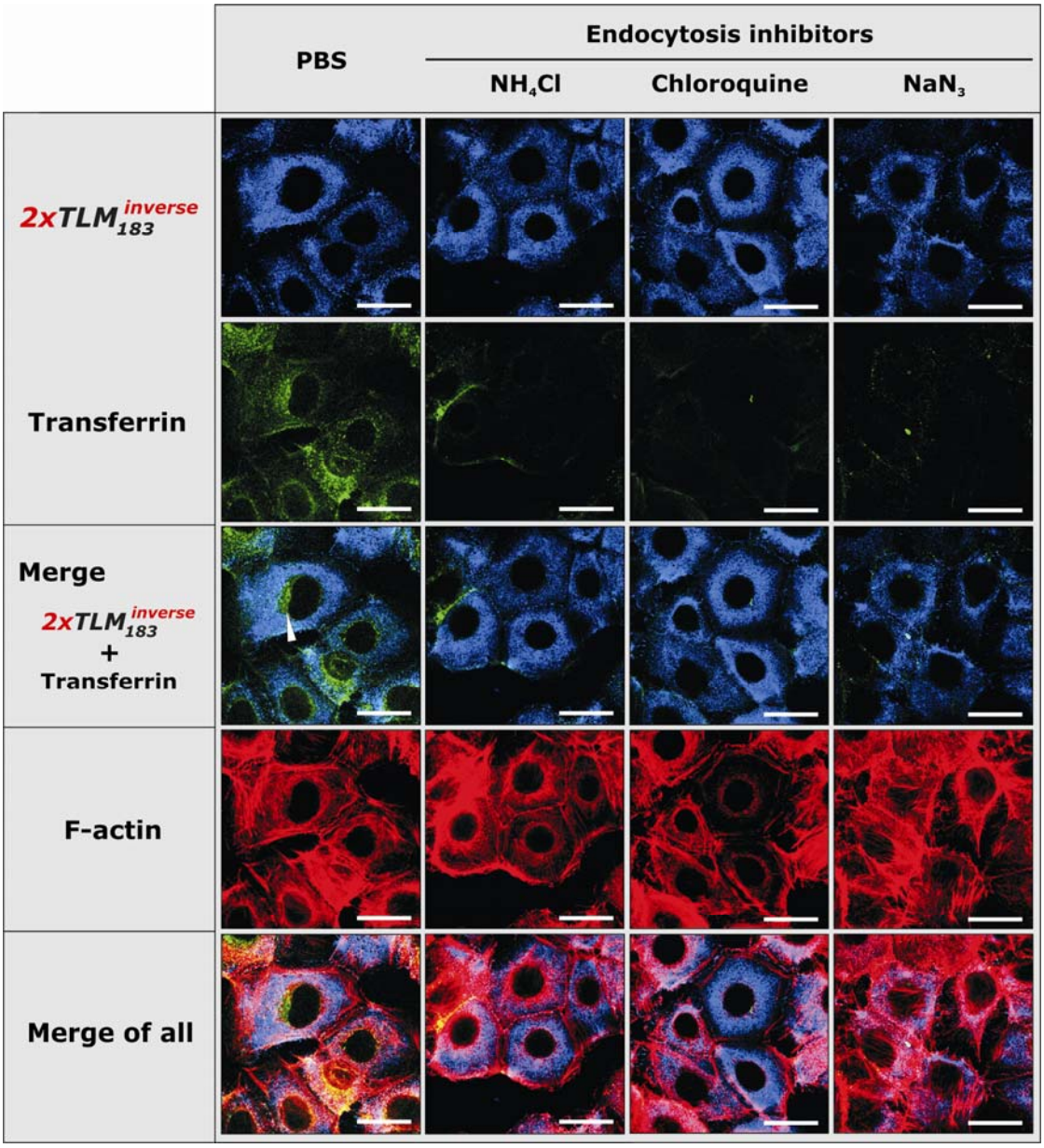


Figure 27: Non-endocytotic uptake of TLM capsids

Huh7 cells treated with endocytosis inhibitors were pre-incubated for 45 min with 15 mM ammonium chloride or 0,2 mM chloroquine or 15 mM sodium azide. All cells were then incubated for 45 min with inverse 2xTLM$_{183}$ (20 nM) capsids and in case of the treated cell with the corresponding endocytosis inhibitor. Fluorescein-labeled human transferrin (20 nM) serves as positive control for endocytotic uptake. After a washing step the cells were fixed, immunostained and visualized with a CLSM. F-actin was stained by TRITC-labeled Phalloidin and serves as an intracellular marker. The non-colocalized distribution (white arrow) clarifies the overlay (merge) of TLM capsid and transferrin staining in the middle. Intracellular localization of TLM capsids is shown in merged images in the bottom row (scale bar: 50 µm).

To confirm the localization of proteins inside the cell, their uptake and intracellular localization was examined by confocal microscopy. In cells not treated with endocytosis inhibitors (PBS) transferrin shows a predominantly punctuated pattern of fluorescence inside of endocytotic vesicles at the cytoplasmic membrane, inside the cytoplasm and a strong signal next to the nucleus.
For TLM capsids a bright fluorescence signal in the cytoplasm as well as in the perinuclear region was observed in almost all cells (>90%). The majority of TLM capsids are not colocalized with transferrin (**Figure 27**, white arrow), suggesting that they are not sequestered within endocytotic vesicles.
The role of intracellular pH on the internalization of TLM capsids was investigated using common neutralization agents. An endocytotic mediated uptake requires acidification of the late endosomal and lysosomal compartments. This can be blocked by ammonium chloride which prevents the acidification by its high buffer capacity. Incubation of cells with the weak base NH_4Cl (15 mM) is often used to block the cytoplasmic delivery of viruses and toxins that use the endocytic pathway to gain access to the cytoplasm [186]. This procedure caused a significant reduction in the pH-sensitive uptake of transferrin [187] but did not affect the translocation of the capsids.
Moreover, treatment with 0,2 mM chloroquine (acting also as a weak base and therefore as an endocytosis inhibitor) had no effect on TLM capsid translocation. Control experiments suggested that the luminal pH of endosomes was modified, because transferrin uptake was blocked.
Beside the endocytosis-independence an energy-independent translocation mechanism has been suggested. Experiments in which the cellular ATP pool is depleted by preincubation of the cells with sodium azide (45 min at 37°C resulting in > 80 % reduction in cellular ATP) should inhibit all energy-dependent internalization. ATP depletion significantly reduced the uptake of transferrin in Huh7 cells but - in contrast - did not affect TLM capsid's ability to enter the cell.

III.2.6 Translocation behavior at different temperatures

The structure of biological membranes changes with temperature. The transport through a lipid bilayer can be directly influenced by the temperature [188]. The most commonly used way for proteins to enter living cells is that of receptor-mediated endocytosis. This process is energy dependent and completely abolished when the cells are incubated at 4°C

[189]. Furthermore, at low temperature the lipid tails pack closely with one another and this leads to a decreased fluidity of the membrane.

To investigate whether cell permeability of inverse 2xTLM$_{183}$ capsids (>5000 kDa) is temperature sensitive, the translocation ability was tested at 37°C and 4°C in HuH7 cells (**Figure 28**).

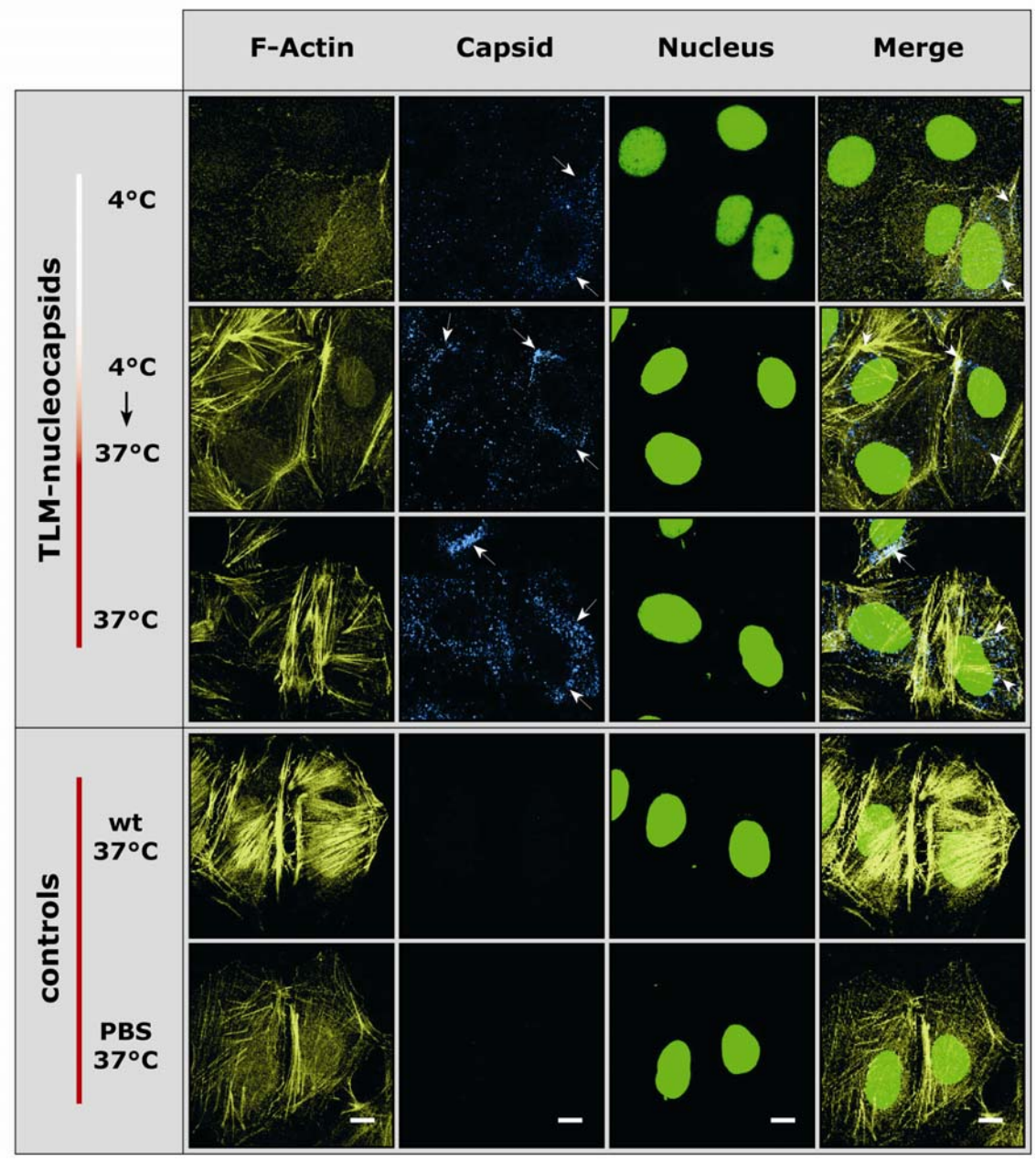


Figure 28: Influence of low temperature on the TLM capsid translocation

CLSM images of Huh7 cells incubated for 45 min with PBS, inverse 2xTLM$_{183}$ or wt capsids (10 nM) followed by a washing step and additional 180 min of incubation with fresh medium. Application of TLM capsids occurs at 4°C or 37°C. After a wash step and replacement of culture medium the temperature stays at 4°C or was increased up to 37°C. PBS and wt capsid incubation takes place continuous at 37°C. F-actin and nucleus staining indicates the structural features and dimensions of the cells (scale bar: 10 µm).

The application of TLM capsids into the culture supernatant occured at 4°C or 37°C for 45 minutes. After having washed the cells and replaced the culture medium, the temperature stays at 4°C or was increased up to 37°C for additional 180 minutes. PBS and wt capsid incubation was performed continuously at 37°C.
In case of the controls (wildtype capsids and PBS at 37°C) no uptake and intracellular signal are detectable. In contrast, the TLM capsids at 37°C translocate into the cytoplasm. At 4°C a significant reduction of the amount of internalized TLM capsids was observed. Capsids are bound to the outer membrane (white arrows), however.
Only a small amount of TLM capsids was found inside the cells (~20% - in comparison to control). After having washed the cells and increased the temperature from 4°C to 37°C membrane bound TLM capsids are able to translocate into the cytoplasm during the 180 minute incubation period.
The fluidity of the cytoplasmic membrane of 4°C allows only a small amount of cell permeable particles with a size around 30 nm to translocate into cells.

III.2.7 Model of cell permeable capsids

Taken together these results leads to the model illustrated in the figure below. Cell permeability was achieved by fusion of inverse orientated double TLM via linker amino acids at the N-terminus of full-length hepatitis B capsid protein. The expression of recombinant core protein in bacteria or insects cells leads to fully assembled capsids with the TLM exposed on their surface. The structure and functionality of recombinant capsids is not affected by the modifications. Only the TLM capsids show cell permeability, therefore they are able to pass the outer membrane of different cell types and can translocate rapidly into the cytoplasm. In a time period of 60 minutes under physiological conditions the fully assembled TLM capsids accumulate in the perinuclear region of living cells but do not enter the nucleus.

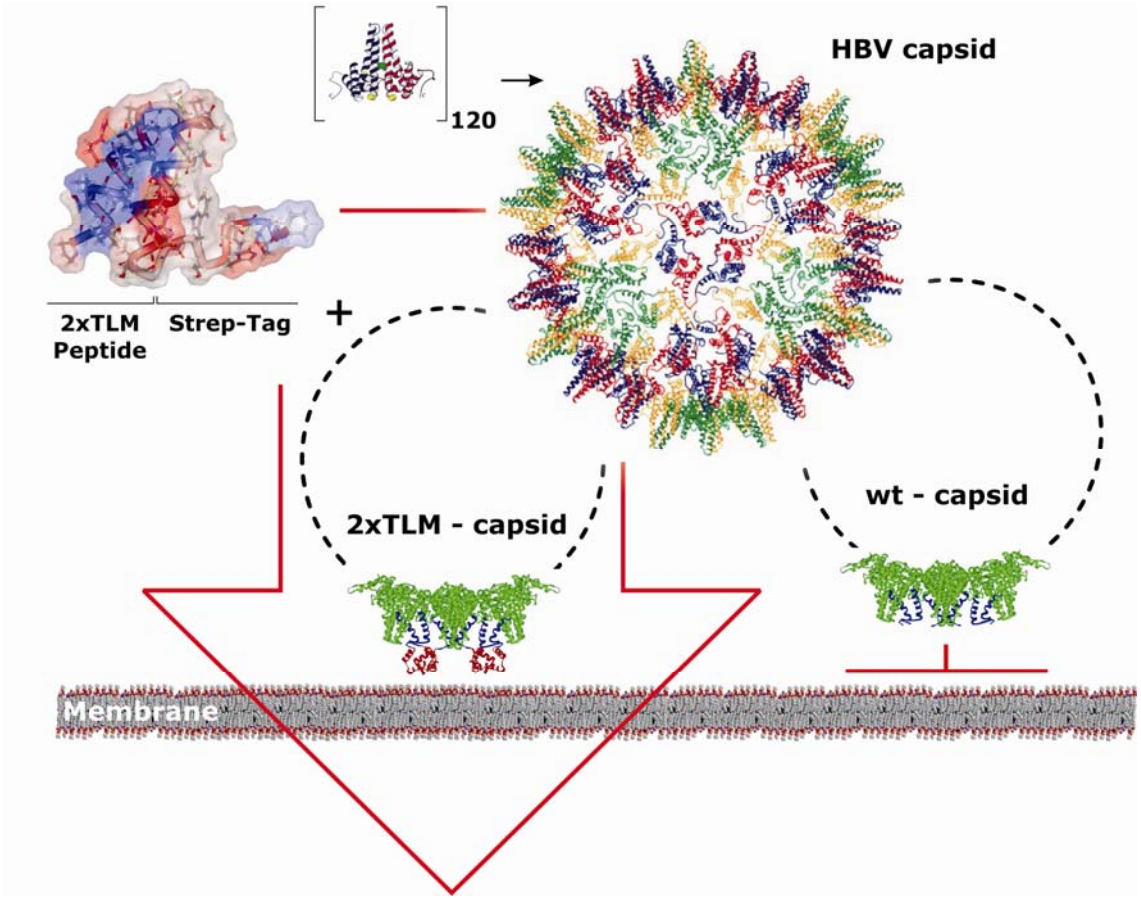


Figure 29: Model of cell-permeable capsids.
120 wildtype or recombinant HBV core protein dimers assemble during the expression in bacteria or insect cells to complete icosahedral particles. Molecular modelling of TLM capsid (left): inverse 2xTLM$_{183}$ peptide (red) is fused by a linker (blue, consisting of 8 aa Strep-tag and 20 aa precore) to the N-terminus of HBV capsid and of the wt capsid (right, wt$_{183}$): only the linker (blue) is fused to the N-terminus. Only the capsid on the left hand featuring the N-terminal fused 2xTLM peptide (inset shows a model of the inverse 2xTLM together with the 8 aa Strep-tag) is able to pass the outer membrane and to translocate into the cytoplasm of a cell.

III.2.8 Specific *in vivo* packaging of nucleic acid into capsids

In case of the *E. coli* system packaging of DNA into capsids can be performed only by *in vitro* dissociation of the capsids and subsequent reassociation in the presence of the cargo DNA. Due to the low expression rate of full-length core protein produced in bacteria as well as the low efficiency and the cost-value ratio of the *in vitro* packaging system there was need to develop a new and better packaging system.

Efficient *in vivo* packaging of nucleic acids requires the arginine-rich C-terminal domain of full-length HBcAg [115,118,190]. As it was shown before, Baculovirus infected insect cells are able to produce high amounts of full-length cell permeable and properly assembled core particles. The packaging of nucleic acids into capsids during the production and assembly of capsids is the most convenient way and mimics the natural infection cycle of hepatitis B virus.

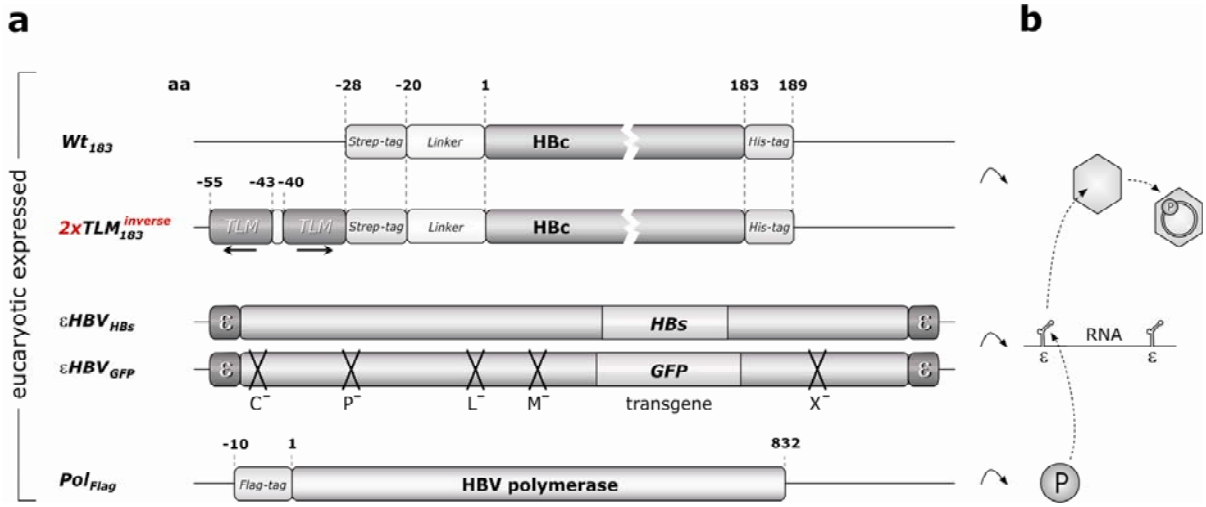


Figure 30: Baculovirus constructs and the principle of the *in vivo* packaging system. **a)** All constructs are based on the HBV ayw genotype. Recombinant baculoviruses containing the eukaryotic constructs (wt_{183}, inverse $2xTLM_{183}$, PolFlag, εHBV-HBs and εHBV-GFP) were used to infect *Spodoptera frugiperda* insect cells (Sf9). The Pol_{Flag} construct was kindly provided by Robert Landford [190]. The εHBV constructs based on the pCMV-HBV30 plasmid (kindly provided by Jong-Keun Jeong [139]). The HBs ORF driven by the liver specific HBs promotor serves as reporter gene. The εHBV_{GFP} construct based on the pCH-S-GFP $(CPLMX)^-$ plasmid (kindly provided by Andreas Untergasser [191]) and provides GFP as reporter gene driven by the liver specific HBs promotor. Every other ORF are disrupted (HBc, Pol, LHBs, MHBs and X protein). **b)** Sf9 cells were triple infected with recombinant baculoviruses coding for the HBV-polymerase (PolFlag), the recombined capsid (wt_{183} or inverse $2xTLM_{183}$) and the packaging plasmid (εHBV-HBs or εHBV-GFP, harboring two of the ε encapsidation signals and a reporter gene). The HBV-polymerase first interacts with encapsidation signals of the packaging plasmid RNA and then the complex is recognized by the core protein and packaged into the assembled capsid.

In vivo packaging of mRNA into capsids and subsequent reverse transcription into DNA requires an ε-motive on the RNA in *cis* and the presence of HBV polymerase in *trans* [192,193]. Using the Sf9 insect cells *in vivo* packaging can be performed. To establish an in vivo packaging system Sf9 cells were triple infected with recombinant baculoviruses encoding for wt or TLM-core protein, for HBV-polymerase and for a RNA harboring ε-encapsidation signals close to its ends (**Figure 30**). This RNA

contains the coding sequence for SHBs or GFP which serve as marker genes and can be easily detected by immunofluorescence microscopy or commercial surface protein (HBsAg)-specific ELISA systems. Packaging of RNA and subsequent conversion into DNA requires core particle associated reverse transcriptase (RT) activity of the HBV polymerase. In order to establish the triple infection of insect cells, the titer and infectivity as well as the optimal ratio of the used Baculoviruses were tested in insect cells by immunofluorescence and flow cytometry (data not shown).

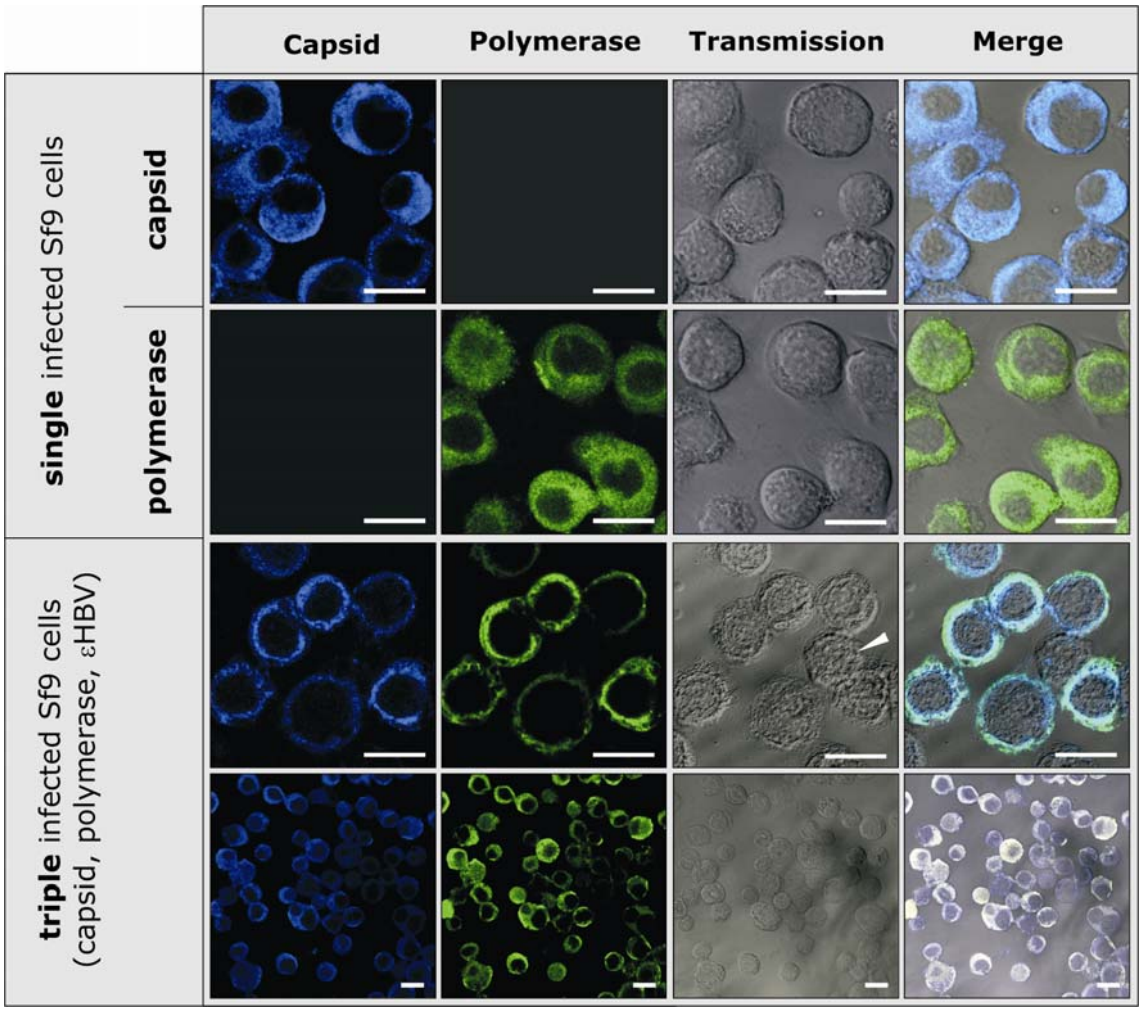


Figure 31: Baculovirus infection of Sf9 cells to produce nucleocapsids.
CLSM images of Sf9 cells infected with one (single) or three (triple infection) recombinant baculoviruses coding for full-length HBV capsid, HBV polymerase or the packaging plasmid (εHBV). To detect cores the monoclonal antibody (MAb3120) and for detection of Flag-tagged polymerase the monoclonal anti-Flag M2 antibody was used. Monitoring of infection with recombinant baculovirus coding for the packaging plasmid (εHBV) is impossible. The reporter gene is driven by an upstream liver specific promotor. Therefore no expression of reporter genes can be observed in insect cells (scale bar: 50 µm).

III.2.8.1 HBV polymerase is packaged together with nucleic acid into nucleocapsids

To analyze whether Sf9 cell-derived nucleocapsids indeed harbor HBV polymerase with reverse transcriptase (RT) activity a product enhanced reverse transcriptase (PERT) assay was performed (**Figure 32**). According to the principle of this assay, the virus specific enzyme (reverse transcriptase) mediates the conversion of a synthetic RNA to cDNA, the cDNA being detected and quantified by TaqMan real time PCR.

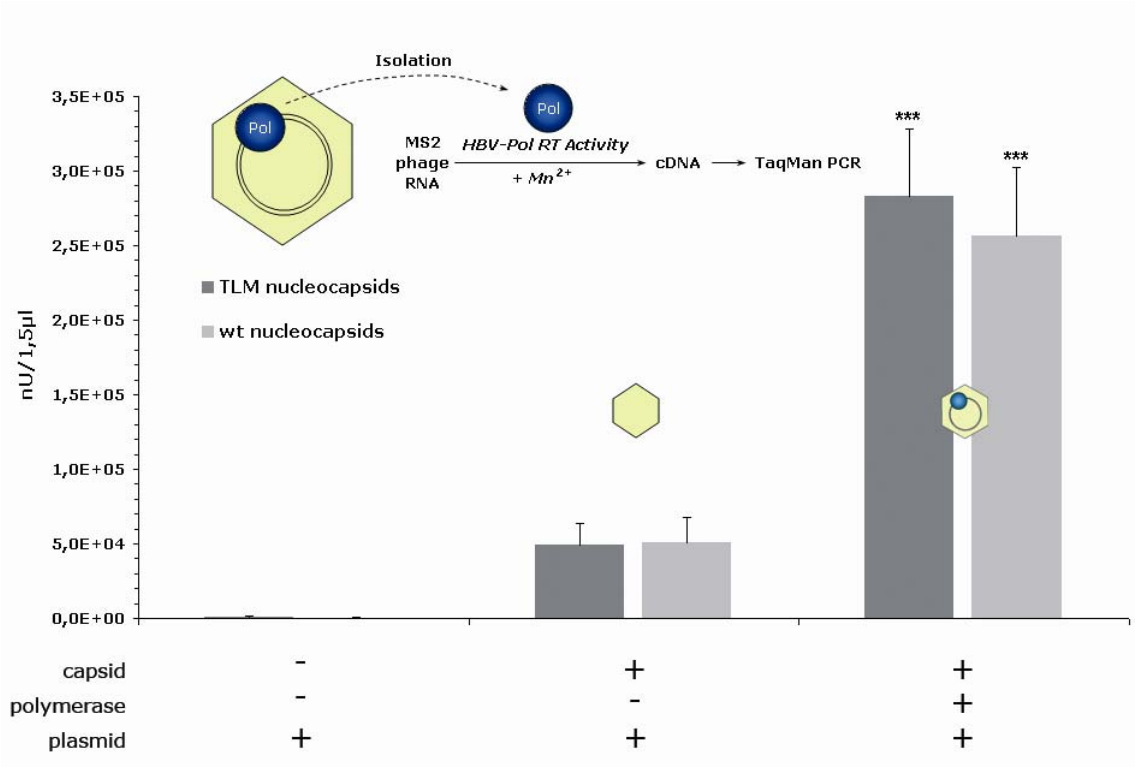


Figure 32: Packaging of active HBV polymerase into nucleocapsids.
TLM and wt nucleocapsids packaged with DNA and HBV polymerase were isolated from triple infected insect cells by affinity and size exclusion chromatography. Activity of packaged HBV polymerase was determined by measurement of reverse transcriptase activity (RT). To this end the product enhanced reverse transcriptase (PERT) assay was performed. Manganese was used as bivalent metal ions in the reaction buffer. Double infected Sf9 cells (the capsid, necessary for the first step of purification, or the polymerase encoding baculovirus was omitted) serves as control.

Purified nucleocapsids from triple infected Sf9 cells were used for PERT assay. As a control double infected Sf9 cells were used. These cells do not express the Strep-tagged capsid, necessary for the first step of purification or the polymerase, necessary for *in vivo* packaging of nucleic acids. The assay revealed that in case of the particles derived from the triple infected cells - compared to the control cells - a significant reverse transcriptase activity could be measured. This data indicates that indeed physiologically active HBV Polymerase is packaged into Sf9 derived nucleocapsids.

III.2.8.2 Expression, purification and characterization of nucleocapsids

The triple infection of Sf9 cells with baculoviruses coding for the capsid, the polymerase and a packaging plasmid leads to the expression of all components necessary for an *in vivo* packaging system according to the life cycle of HBV. In comparison to the bacterial expressed capsids and the *in vitro* packaging of DNA by dis- and reassembly of HBc particles the triple infection of Sf9 cells provide an elegant system to generate nucleocapsids in one step. To prove the packaging of nucleic acid by this system the nucleocapsids were first purified by affinity and size exclusion chromatography (**Figure 33a**). The elution profile indicates that the yield of the assembled capsids is nearly 100% with almost no detectable monomers present.

Selected fractions were than analyzed by silver stained SDS-PAGE (**Figure 33b**) and Western blot respectively (**Figure 33c**). Transmission electron microscopy of nucleocapsid peak fractions was performed. The size and structure of generated nucleocapsids corresponded to the hepatitis B wildtype nucleocapsids (**Figure 33 inlet**).

Based on these results, the next sets of experiments addressed whether the purified particles indeed harbor DNA. To investigate this question, purified TLM or wt nucleocapsids isolated from Sf9 cells were subjected to DNase treatment to remove adhering DNA from the surface of the particle while the packaged DNA is protected. To deactivate the DNase and to set free the nucleic acid free from the core particles proteinase K was added. The packaged DNA was isolated from the reaction mixture as described in the methods section.

The isolated DNA was subjected to PCR analysis by amplifying a SHBs-specific sequence. To exclude that positive signals are due to contamination with recombinant baculovirus an additional PCR amplifying a sequence specific for the baculovirus polyhedrin promoter was performed (**Figure 33d**). The PCR revealed that in nucleocapsids SHBs-specific DNA was detectable while no Baculovirus DNA was present. In order to quantify the amount of packaged DNA TaqMan PCR was performed. The real-time PCR indicated that 1×10^6 DNA molecules per µg purified TLM and wt nucleocapsids were detectable. Correlation of this data with the amount of core particles used in these experiments indicates that about 0,1‰ of the TLM nucleocapsids isolated from this expression system specifically harbor a copy of the marker gene.

Taken together this data demonstrates that efficient DNA packaging into core particles can be achieved by triple infection of insect cells.

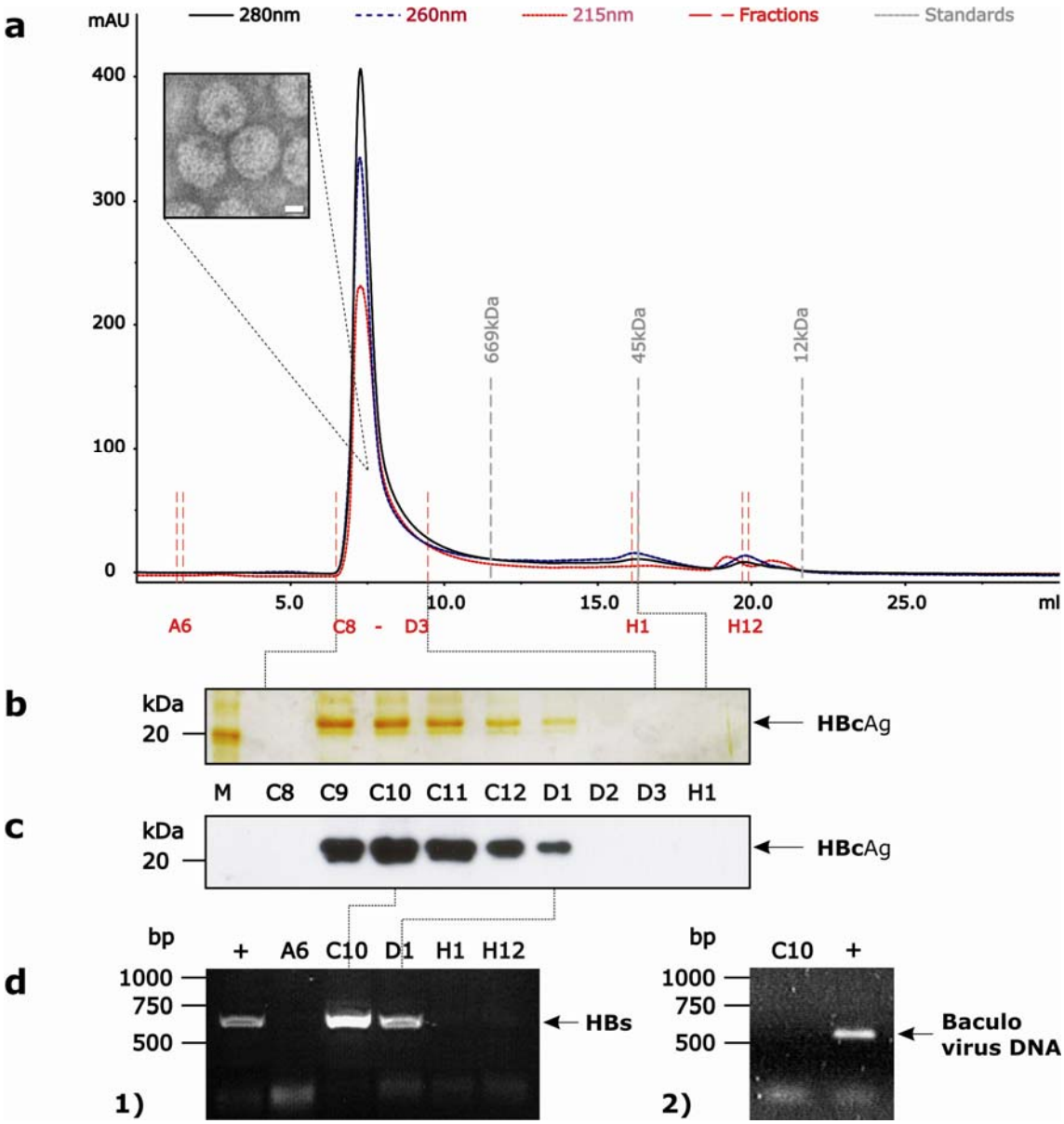


Figure 33: Purified TLM <u>nucleocapsids</u> expressed from triple infected insect cells harboring specific DNA.

a) Elution profiles of 2xTLM$_{183}$ core particles packaged with DNA and isolated from triple baculovirus infected insect cells. For capsid purification a calibrated Superose 6 gel filtration column was used; standards: Thyroglobuline (669kDa), Ovalbumin (45kDa) and Cytochrom C (12kDa). **Inlet)** Core peak fraction was analyzed by transmission electron microscopy (negative stain, scale bar: 10nm). **b)** Selected fractions were analyzed by silver staining and **c)** Western blotting of a 15% SDS gel. The monoclonal antibody (MAb16990) was used to detect the HBcAg. **d1)** To prove packaging of DNA into cores the isolated particles were subjected to DNase followed by proteinase K treatment and protected DNA was analyzed by HBs specific PCR. **d2)** Control PCR to exclude baculovirus contamination.

III.2.9 TLM nucleocapsids are cell permeable and translocate as fully assembled particles into Huh7 and primary human hepatocytes

Rabe and coworkers described that the maturation of capsid, the existence of polymerase and packaged viral genome influences the characteristics of the hole assembled capsid [169]. To prove the ability of TLM nucleocapsids to translocate into cells in the same way as it was observed in case of empty TLM capsids Huh7 and PHH were incubated with TLM nucleocapsids.
Primary cultured hepatocytes represent a very helpful tool to perform *in vitro* studies of liver diseases. PHH have been used as one of the most important target cells in basic and clinical research. It would be of great interest to make the efficient transfer of genes into hardly transfectable primary cell cultures possible.
A crucial prerequisite for the concept of a TLM nucleocapsid mediated gene transfer is the cell permeability of these nucleocapsids. To address this question liver cells grow for 180 minutes in the presence of TLM nucleocapsids or the corresponding wt control.

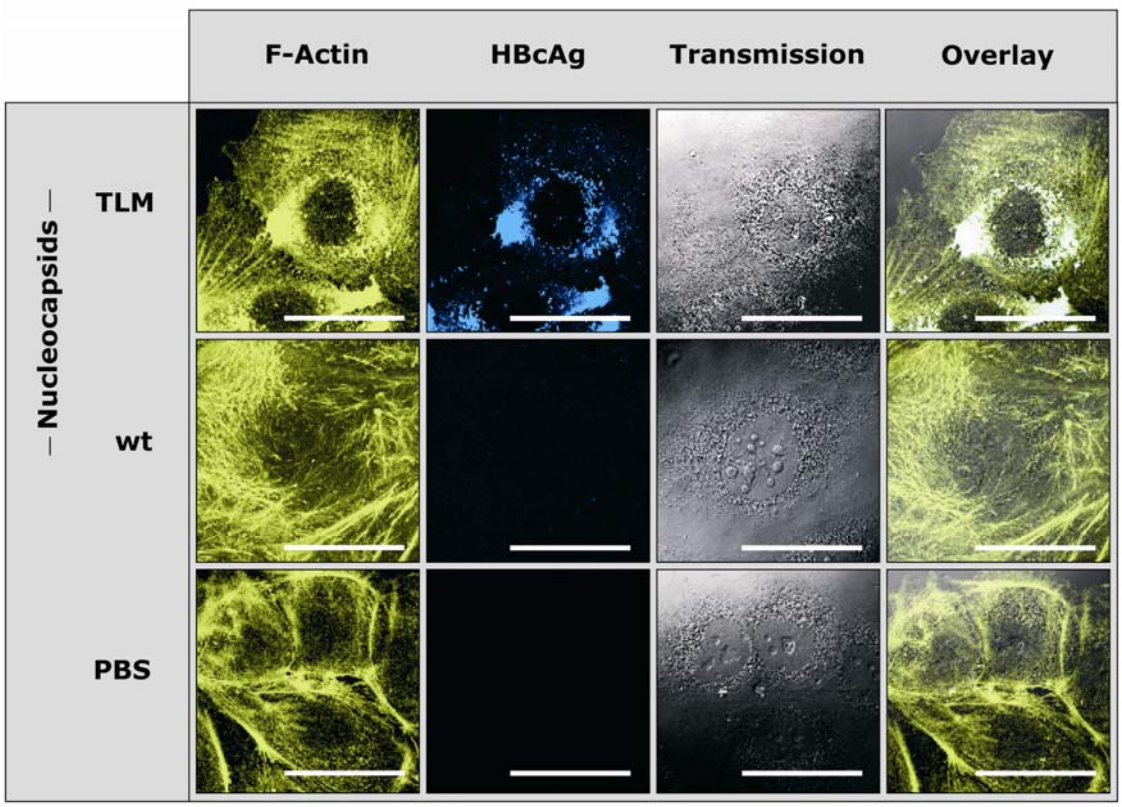


Figure 34: Fully assembled TLM nucleocapsids are able to translocate into Huh7.
CLSM images of Huh7 cells grown for 180 minutes in the presence of PBS, wildtype (wt) and TLM nucleocapsids (20 nM of wt_{183}/ inverse $2xTLM_{183}$). For detection of the nucleocapsids the monoclonal antibody (MAb3120), that selectively recognizes complete particles, was used (scale bar: 50 µm).

The nucleocapsids were isolated from triple infected Sf9 insect cells and diluted in cell culture medium. In the first set of experiments Huh7 cells were incubated. After fixation of the cells internalized nucleocapsids were visualized by the HBV capsid specific antibody (MAb3120) [182]. F-actin as an intracellular marker was visualized by fluorescence-labeled phalloidin. The specimens were analyzed by confocal laser scanning fluorescence microscopy (**Figure 34**).

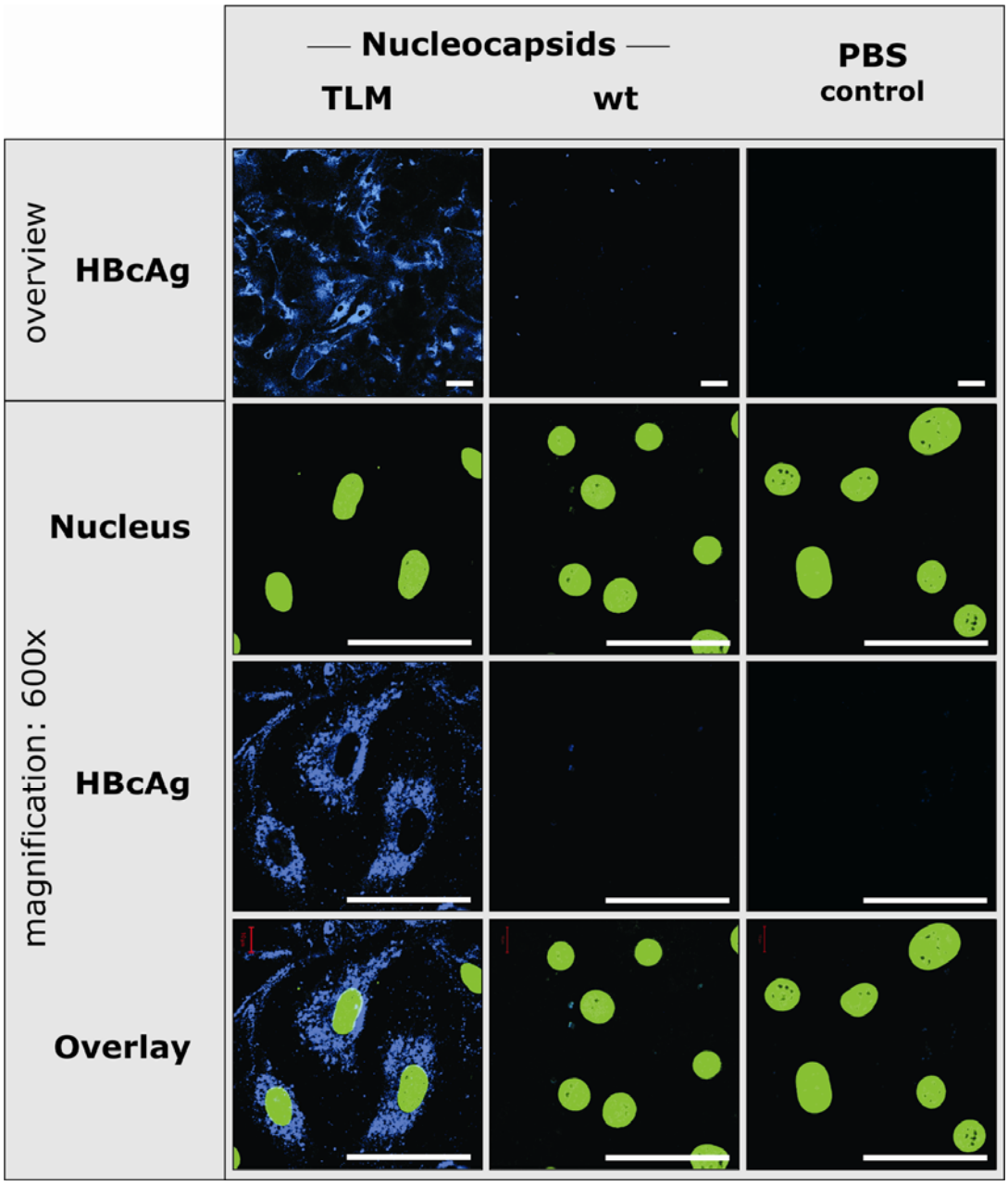


Figure 35: TLM nucleocapsids are able to translocate into primary human hepatocytes.

Primary human hepatocytes (18h after biopsy) were incubated for 180min with wt or TLM nucleocapsids (20 nM of wt_{183}/ inverse $2xTLM_{183}$) or PBS respectively. The nucleocapsids in the fixed cells were detected by using the HBV capsid specific antibody (MAb3120). Nuclei were stained by SYBR green (Scale bar: 50µm).

Confocal analyses revealed that in case of cells that were grown in presence of full length wt nucleocapsid no capsid-specific staining could be observed inside the cells. In case of Huh7 cells grown in the presence of TLM nucleocapsids, a significant staining of the cytoplasm especially in the perinuclear region but not in the nucleus was observed reflecting the translocation of intact particles across the plasma membrane. Fully assembled nucleocapsids were not equally distributed in the perinuclear region of cells after 180 minutes incubation. The nucleocapsids show a cap-like distribution around the nucleus.

The second set of experiments was performed with freshly isolated primary human hepatocytes. As expected, the TLM nucleocapsids were able to translocate into almost all PHH (**Figure 35**). The upper images of PHHs taken with a lower magnification give an impression of the high translocation efficiency of TLM nucleocapsid. Confocal laser scanning microscopy images of higher magnification show the same bright cytoplasmic staining next to the nuclear membrane as observed in case of the Huh7 cells.

Moreover, cell permeability of the TLM nucleocapsids was shown in a variety of hepatic and non-hepatic cell lines i.e. Hela-, fibroblasts- and dendritic-cells (data not shown). This data demonstrates that TLM nucleocapsids indeed translocate as assembled particles across the membrane. The observation that almost all cells - even primary cells - exposed to the TLM nucleocapsids are stained, underlines the efficiency of the TLM mediated transport.

To verify the results of previous research ultra thin sections of Huh7 cells incubated with TLM nucleocapsids were analyzed by transmission electron microscopy (**Figure 36**). Cells were incubated for 45 min and washed with PBS to remove free nucleocapsids. In the early stage of translocation (up until 45 min) many assembled TLM nucleocapsids are detectable on the cell surface.

In spite of having performed a washing step nucleocapsids are detectable on the cell surface. They show a strong interaction with the cytoplasmic membrane components (upper picture, black and white arrows). After 180 minutes the nucleocapsids are still properly assembled and accumulate next to the nuclear membrane and nuclear pore complex (lower picture). The distribution of capsids in the cytoplasm of ultra thin sections is equivalent to the distribution observed by confocal laser scan microscopy. This data show that cell permeable TLM nucleocapsids translocate as complete particles into the cytoplasm and that intracellular trafficking towards the nucleus takes place.

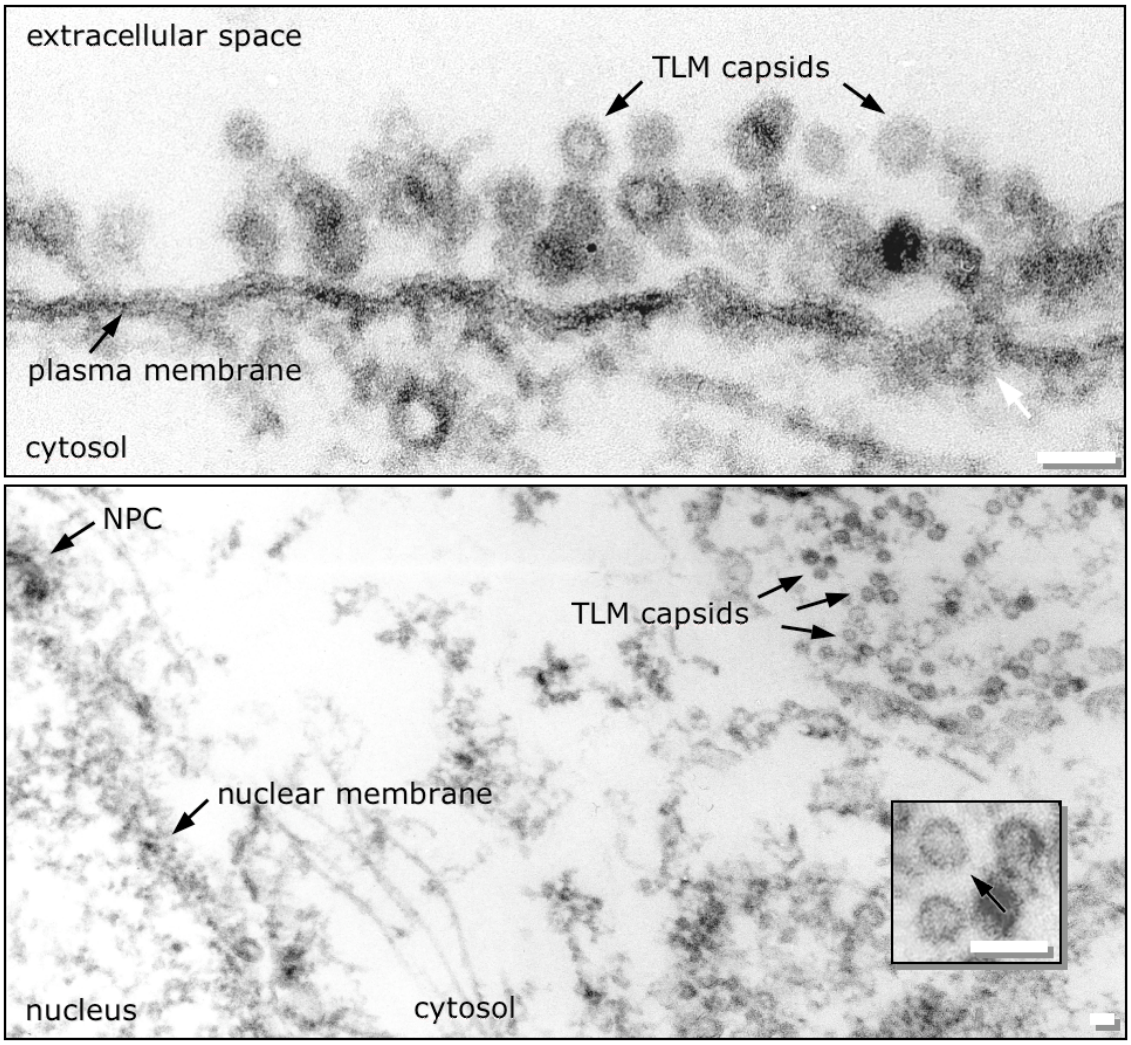


Figure 36: Fully assembled cell permeable TLM nucleocapsids translocate into the cytoplasm of Huh7 cells.

Transmission electron microscopy (TEM) images (scale bar: 50 nm) of Huh7 cell ultra thin section. **Upper picture)** Huh7 cells were incubated for 45 minutes with highly purified TLM nucleocapsids (inverse 2xTLM_{183}, 20 nM) expressed in insect cells. **Lower picture)** Section next to the nuclear membrane (with nuclear pore complex, NPC) after 180 minutes (**Inlet** shows a higher magnification of the TLM nucleocapsids located in perinuclear cytosol).

III.3 EFFICIENT GENE TRANSFER BY CELL PERMEABLE TLM NUCLEOCAPSIDS

The experiments described above demonstrate that TLM core particles are indeed cell permeable, that nucleic acids can be packaged into these particles and inside of the cells a directed transport towards the nucleus takes place. These data suggest that all prerequisites for gene transfer mediated by TLM-nucleocapsids should be fulfilled. Therefore, it was finally investigated whether these particles indeed can be used for gene transfer.

Huh7 cells were grown in the presence of wt or TLM nucleocapsids harboring a SHBs encoding sequence as marker gene. An analysis of marker gene expression was performed either by immunofluorescence microscopy or HBsAg specific ELISA. For immuno-fluorescence microscopy cells were fixed 48 h after incubation with TLM nucleocapsids or the respective controls and stained using an HBsAg-specific antiserum (**Figure 37**). The immunofluorescence clearly demonstrates that cells, which were exposed to TLM nucleocapsids, the marker gene was delivered in almost all of the cells, visualized by the HB**s**Ag-specific staining. In case of cells grown in the presence of wt nucleocapsids no significant gene transfer could be observed.

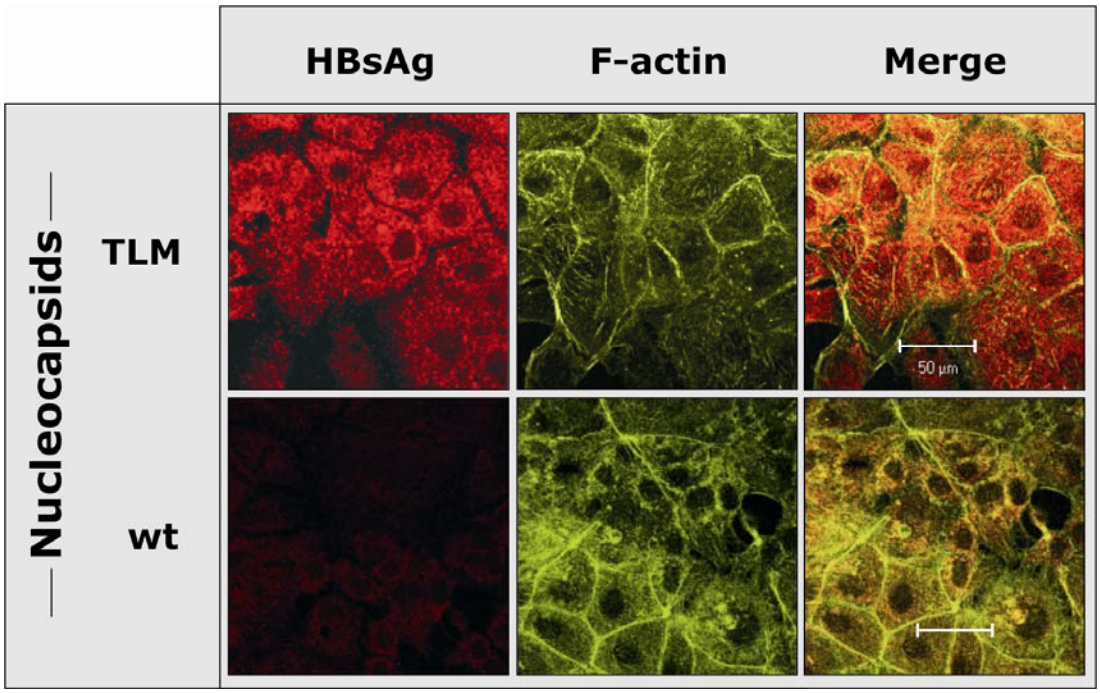


Figure 37: Efficient gene transfer by cell permeable TLM nucleocapsids harboring a marker SHBs gene.

CLSM images of Huh7 cells incubated for 180min with TLM and wt nucleocapsids (genomic equivalent of 10^2 -10^3). Cells were fixed 48h past incubation. SHBs synthesis that served as marker for gene transfer is shown in red (polyclonal anti HBsAg antibody, Dako). Cytoskeleton (F-actin) was visualized by staining with FITC-phalloidin (scale bar: 50 µm).

The expression of reporter gene (HBsAg) shown by immunofluorescence analysis was confirmed by HBsAg-specific ELISA. In the supernatant of liver cells, incubated with TLM nucleocapsids, significant amounts of the secreted protein HBsAg could be detected. This demonstrates that successful gene transfer (**Figure 38a**) has occurred.

The performed experiments show that TLM nucleocapsids can even translocate into primary human hepatocytes. All currently known

transfection methods are not suitable to transfect these cells with a mentionable efficiency. In contrast by using TLM nucleocapsids it was possible to develop a gene transfer system for these cells.

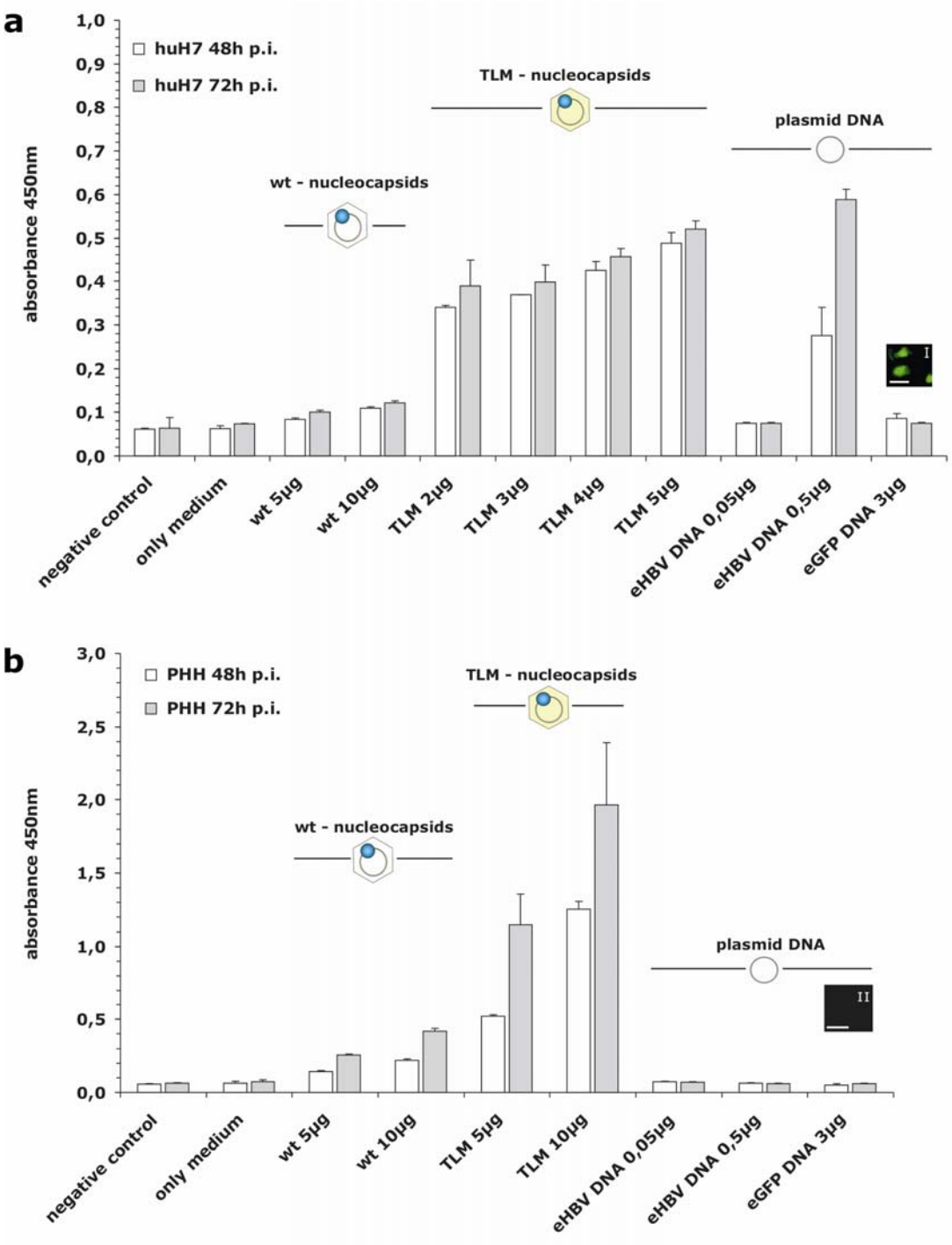


Figure 38: Gene transfer in HuH7 cells and primary human (PHH) hepatocytes
a) HuH7 cells and **b)** 18h old primary human hepatocytes were incubated for 180min with wildtype or TLM nucleocapsids (wt_{183} / inverse $2xTLM_{183}$). 100µg nucleocapsids are correspond to a genomic equivalent of 10^2 - 10^3. Control cells were transfected with the packaging plasmid harboring the HBsAg gene or eGFP (Inlet I and II shows eGFP fluorescence, scale bar: 50 µm). After 48h and 72h samples were collected for the anti HBsAg ELISA (Dade Behring).

The HBsAg-specific ELISA clearly demonstrates that in primary human hepatocytes transfected using a conventional lipofection approach or wt nucleocapsids no significant HBsAg-production occurred.
However, in cells that were incubated with TLM nucleocapsids strong expression of the marker gene was observed, reflecting an efficient gene transfer (**Figure 38b**). By increasing the amount of TLM nucleocapsids used a higher ELISA signal was measured. After 72 h the amount of secreted HBsAg reached its maximum.
Using nucleocapsids which harbor GFP as a reporter gene, the same results were obtained for gene transfer into primary human hepatocytes (**Figure 39**). A higher concentration of TLM nucleocapsids (20 nM) causes a bright GFP fluorescence in almost all cells (>90%). The CLSM images of 10 nM concentrated TLM nucleocapsids show a decreased amount of PHH expressing GFP. No significant GFP signals were detected in cells incubated with 20 nM wt nucleocapsids or PBS. A GFP positive control is missing simply because of the lack of a commercialized transfection system for PHH.

This data demonstrates that based on cell permeable TLM nucleocapsids a novel tool for efficient gene transfer into hardly transfectable primary human hepatocytes has been established.

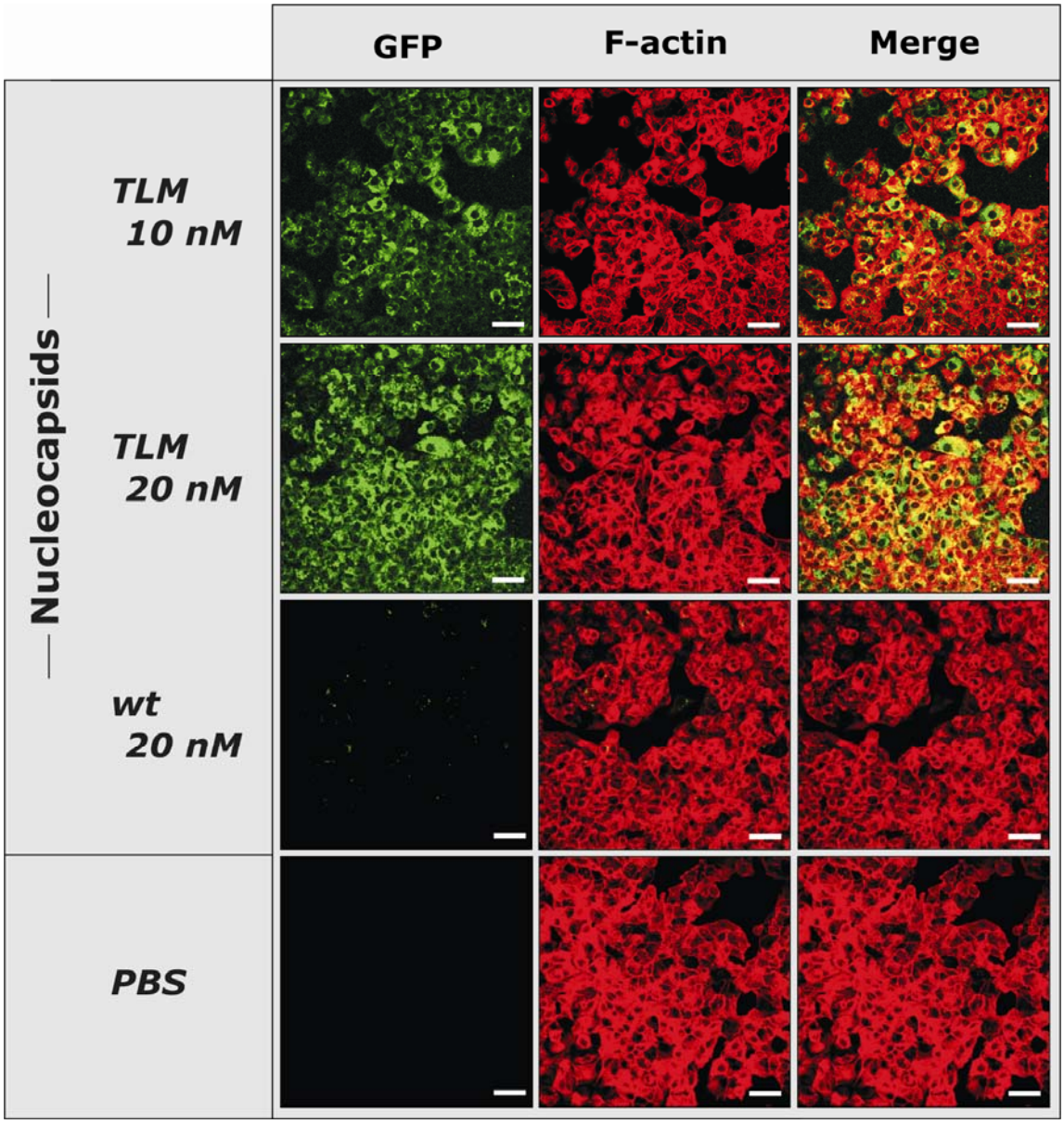


Figure 39: Efficient gene transfer into primary human hepatocytes

21h old primary human hepatocytes were incubated for 240min with wildtype or TLM nucleocapsids (wt_{183} / inverse $2xTLM_{183}$). 20 nM nucleocapsids are equivalent to a genomic equivalent of 10^2 -10^3. Control cells were transfected with the packaging plasmid harboring the GFP reporter gene (scale bar: 50 µm). After 48h cells were fixed, the F-actin as an intracellular marker was stained (Phalloidin-TRITC) and confocal laser scan microscopy analyses was performed.

III.4 HEPATITIS B VIRUS BIOLOGY

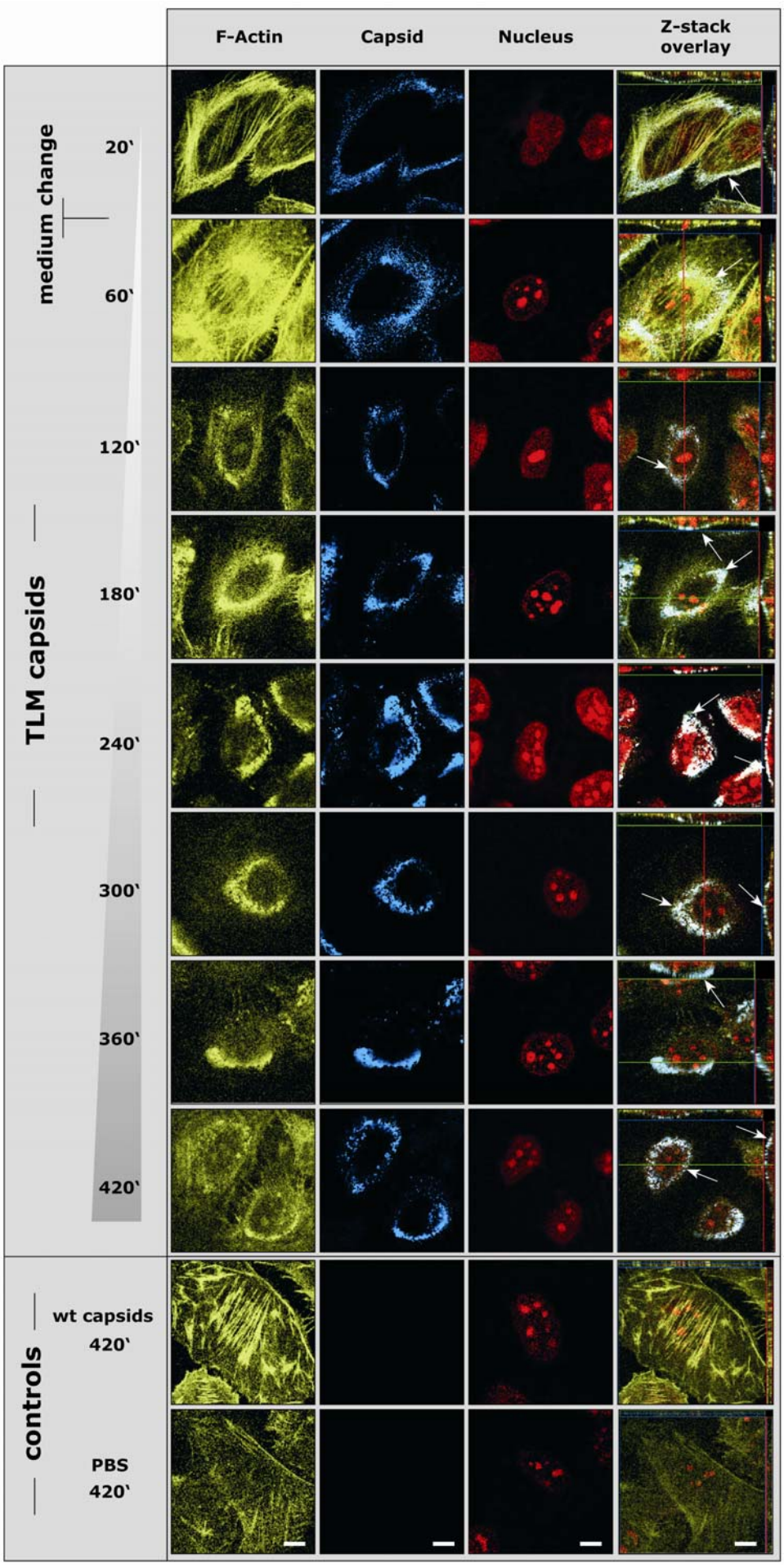
Several human hepatocyte cell lines retaining some of the hepatocyte markers are available that support HBV transcription and replication upon plasmid transfection. However, even these permissive cell lines are not susceptible to HBV infection. Therefore very little information exists regarding the post-entry events in the hepadnaviral life cycle. The viral genome must be transported to the nucleus, where it is transcribed. After escape from the endocytotic vesicle, the core particle (encapsulating the viral genome) is a prominent candidate for mediating genome transport through the crowded environment of the cytoplasm towards the nucleus [21]. Along the same line, to ensure productive infection, the virus likely engages a nuclear import machinery following exit from the endocytic vesicle [194]. The core protein contains nuclear localization signals (NLS) at its highly basic C terminus [92] and has been shown to bind the hepatocyte nuclear membrane [93]. *Escherichia coli*-derived core particles were shown to directly bind the NPC in a core phosphorylation and importin-dependent manner [94].
Based on the described cell permeable HBV nucleocapsids the early post-uncoating steps of HBV infection were investigated in the following experiments.

III.4.1 Trafficking of nucleocapsids towards the nucleus

To study intracellular trafficking of cell permeable TLM nucleocapsids in more detail kinetic experiments were performed. Huh7 cells were incubated for 45 min with TLM nucleocapsids without affecting the integrity of the cell followed by a medium change. Then the cells were fixed every 60 min up to 420 min (**Figure 40**). For detection of TLM nucleocapsids confocal laser scanning microscopy was performed.

Figure 40: Cytoplasmic trafficking of fully assembled cell permeable TLM core particles.

Confocal laser scanning immunofluorescence images of the time course of internalized TLM nucleocapsids. Cells were incubated for 45 min with cell permeable nucleocapsids (20 nM) followed by a washing step and an additional incubation time with fresh medium. Cells were fixed every 60 min up to 420 min. The cytoskeleton was stained with FITC labeled Phalloidin, the nucleocapsids were detected by a polyclonal (Biomeda) as well as a monoclonal antibody against HBcAg (MAb3120, data not shown). The nucleus was stained by Toto-3. The overlay image shows the x, y optical section through the center of the cell and x, z and y, z projections (right and upside) of this confocal image (scale bar: 10 µm).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Nucleus
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PBS
420`

The TLM nucleocapsids rapidly translocate across the plasma membrane (already after 5 minutes) from the medium into almost all cells, whereas no significant internalization was seen in case of wild type nucleocapsids, lacking the TLM. The major fraction of internalized cores moves as intact particles from the cytoplasm towards the nucleus. From 120 min to 420 min the capsids show a rim like staining around the nucleus. Even after 60 hours post incubation complete capsids or HBcAg dimers could not be detected inside the nucleus. During trafficking to the nucleus a remarkable colocalization of fully assembled nucleocapsids with parts of the cytoskeleton (F-actin) was observed. Moreover, a significant reorganization of F-actin filaments and its architecture was observed when comparing early (20 and 60 min) with late time points (240 and 300 min).

To analyze whether the observed re-organization of the cytoskeleton was caused by direct or indirect interaction of HBV capsids with cytoskeleton components, wt and TLM nucleocapsids were subjected to a protein-protein interaction assay (**Figure 41**). In this initial experiment the nucleocapsids derived from insect cell expression system or PBS which serves as negative control were immobilized on affinity chromatography columns. After having performed a Huh7 cell fractionation the cytosol was applied to the columns followed by stringent washing steps.

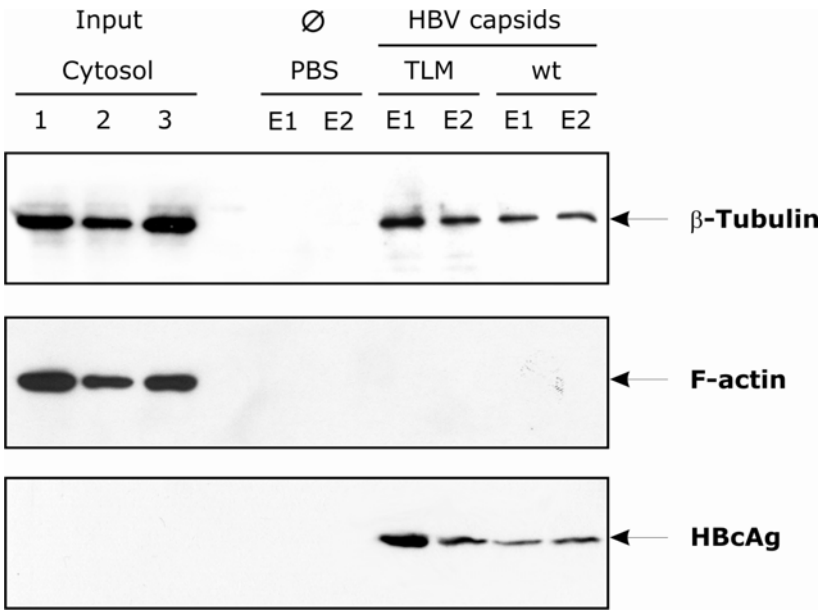

Figure 41: Cytosolic interaction partner of hepatitis B virus capsids

Insect cell derived nucleocapsids (wt_{183} / $2xTLM_{183}$) or PBS as negative control were immobilized on affinity chromatography column (in each case two Strep-tag columns, IBA). Freshly prepared Huh7 cytosol (**Left**: Input for columns (1=PBS), (2=TLM) and (3=wt)) was applied to the column followed by intensive washing steps. **Right**: capsids with interaction partners were eluted from the columns. Elution fractions (E1, E2) were separated by 10-15% SDS-PAGE and immunoblotted with monoclonal antibodies against β-Tubulin (Santa Cruz), F-actin (Sigma) and HBcAg (Chemicon).

To identify potential cytosolic interaction partner of the nucleocapsids the immobilized capsids were eluted. Elution fractions were separated on a SDS-PAGE and immunoblotted. Using this method a strong direct interaction of nucleocapsids with β-tubulin was identified. Whereas, direct binding of F-actin could not be detected in this assay. These first results of the protein-protein interaction assay suggest that the capsid of HBV similar to other viruses interacts with the cytoskeleton [153,195]. The direct interaction with the cytoskeleton or indirect interaction via motor proteins like dynein and dynactin [196] could be essential for the virus capsid transport.

III.4.2 Nuclear import of hepatitis B capsid protein

Many viruses that replicate within the nucleus of nondividing cells use parts of the cytoskeleton for transport of their capsids towards the nucleus and importins to ensure import of their genomes into the nucleus [21]. Because of the limited size of the NPC, capsids of large viruses such as herpes virus, adenovirus, and influenza virus release their genome before the nuclear import. It has been suggested that small viruses such as parvoviruses may enter through the NPC in intact form [197]. With diameters of 32 or 36 nm, the hepatitis B virus (HBV) capsids - analyzed in this study - are close to the size limit of the NPC [168,198]. Sections of HBV infected liver cells show indisputably a nuclear HBcAg staining [199,200]. But little is known about the assembly status of core protein during the infection and inside the infected liver cell nucleus. Both, intact particles or dissociated core dimers are conceivable as HBc conformations during infection and entry of the nucleus. In the absence of an HBV infection system it is hard to investigate the interaction of assembled capsids with intact nuclei. Cell permeable TLM nucleocapsids can be used as a new model system to investigate the post-entry steps of HBV infection.

As it was shown in this study cell permeable nucleocapsids are able to translocate into non affected cells and accumulate in the perinuclear region under physiological conditions. In the experiments performed no capsids were found inside the nucleus. An important question to be answered is whether the TLM nucleocapsids are unable to pass the nuclear membrane.

In living unaffected cells the capsids interact directly or indirectly with cytosolic partners during intracellular trafficking. The binding of cytosolic proteins at the surface of fully assembled capsids could interfere with the functionality of inverse 2xTLM. In an attempt to clarify if TLM capsids have the ability to pass the nuclear membrane intact nuclei were isolated followed by direct incubation with the nucleocapsids (**Figure 42**).

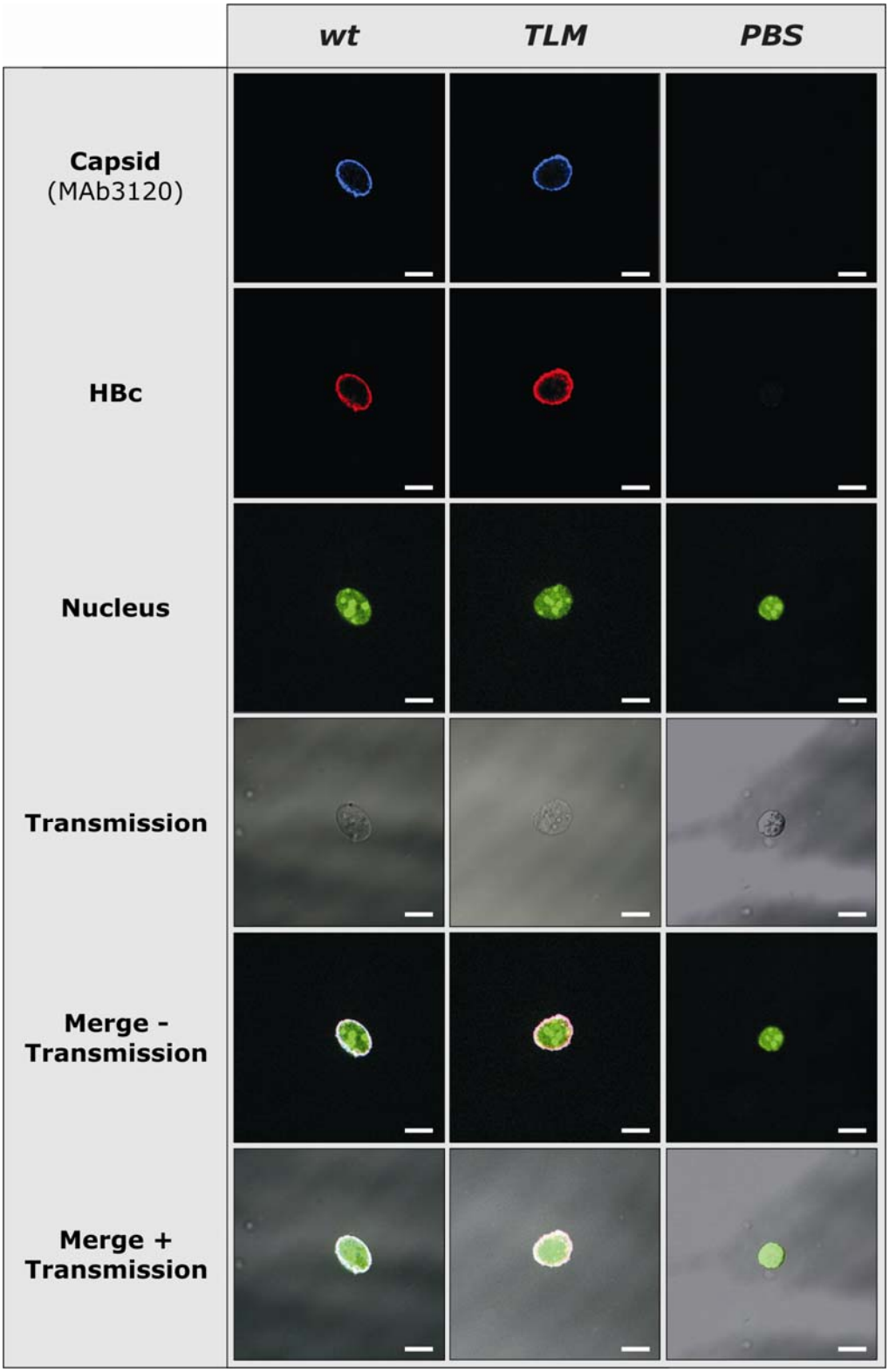

Figure 42: Nucleocapsids are unable to enter isolated liver cell nuclei.
CLSM images of nuclei isolated from Huh7 cells and incubated for 60 min under physiological conditions with TLM and wt nucleocapsids. Nuclei were fixed and stained by SYBR Green. Nucleocapsids were detected by a polyclonal (anti-HBc, Biomeda) as well as by a monoclonal antibody (capsid specific, MAb3120). The optical sections presented here were localized approximately at the mid-height level of nuclei. Photos obtained by transmitted light microscopy indicate the contours of intact nuclei and the absence of cytoplasmic impurities (scale bar: 10µm).

As it is described in experimental procedures a new protocol for the isolation of intact nuclei from liver cells was developed. To isolate intact nuclei the Huh7 cells were exposed to shear stress. The nuclei were resuspended in physiological buffer followed by incubation with nucleocapsids. After incubation time of 60 minutes the nuclei were carefully washed and a mild fixation was performed with paraformaldehyde.

Neither the monoclonal antibody (MAb3120), specific for fully assembled HBV capsids, nor the polyclonal serum, which recognizes also dimers and monomers of HBcAg were able to detect a significant signal inside the intact nuclei. TLM and wt nucleocapsids interact with the nuclear membrane and showed a typical rim-like nuclear envelope staining, but did not enter the nucleus. The results suggest that the TLM nucleocapsids are unable to translocate into nuclei by passing the nuclear membrane.

Kann and coworkers developed an experimental setting using digitonin-permeabilized cells to observe the nuclear import of hepatitis B capsid protein *in vitro* [169]. Purified HBV nucleocapsids were incubated with cells that had been digitonin-permeabilized to provide access to the nucleus [201]. The permeabilized cells were washed. Thereby they lose the entire soluble part of the cytosol. Incubation of these permeabilized 'cells' lacking their own cytosol with nucleocapsids was performed in the presence of ATP and reticulocyte lysate (**Figure 43**). The bound capsids were then visualized by confocal laser scanning microscopy using HBcAg specific antibodies.

Wildtype and TLM nucleocapsids are able to move unhindered towards the nucleus of permeabilized cells. By using confocal immunofluorescence microscopy the nucleocapsids were detected via a specific polyclonal and a monoclonal antibody (MAb3120, specific for assembled capsids) after 20 minutes in the perinuclear region as well as bound to the nuclear envelope. Neither dimers nor fully assembled capsids are imported into the nucleoplasm. After 60 minutes beside the rim-like nuclear envelope staining, a weak dimer staining inside the nucleoplasm was observed in some cases (white arrows). Finally after 120 minutes almost all cells show a bright nuclear staining for hepatitis B core protein. The monoclonal capsid conformation specific MAb3120 antibody was not able to detect any signals inside the nucleus. Only the polyclonal serum detects capsid protein inside the nucleoplasm. The fully assembled nucleocapsids detected by MAb3120 still show the typical rim-like nuclear envelope staining without exception.

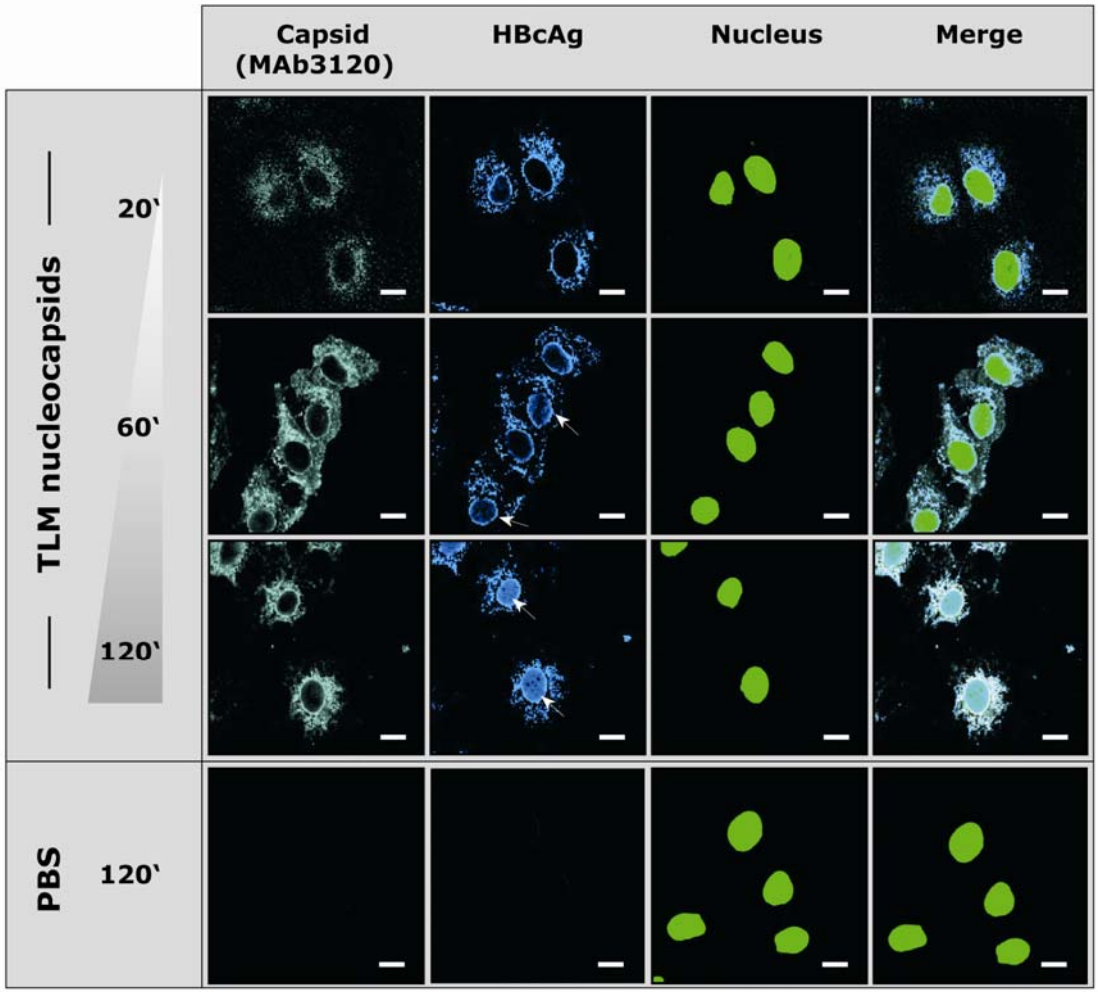

Figure 43: Localization of hepatitis B capsids in digitonin-permeabilized cells
Huh7 cells were permeabilized using digitonin (80 µg/ml) and incubated with purified TLM nucleocapsids (20 nM). Cells were fixed after 20, 60 and 120 minutes incubation time and were immuno-stained using the capsids specific monoclonal antibody MAb3120 and a polyclonal antibody against HBcAg. The optical sections presented were taken approximately at the mid-height level of cells by CLSM. The nucleus was stained by SYBR green. In all samples, ATP and an ATP-generating system were present, and rabbit reticulocyte lysate was used as the source of cytosolic proteins.

More than 90% of used nucleocapsids are indeed fully assembled core particle (see **Figure 33**). However, all factors necessary for disassembling of core particles and import of core dimers into the nucleus are incorporated into the reticulocyte lysate. As it was shown in **Figure 42** the nucleocapsids are unable to pass the nuclear membrane. An import into the nucleoplasm can only occur by passing the NPC.
Taken together, theses findings showed that HBV nucleocapsids interact with the nucleus of permeabilized cells and after 120 minutes only dimers of HBcAg were imported into the nucleoplasm in the presence of ATP and reticulocyte lysate.