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ORIGINAL ARTICLE

Folding properties of the hepatitis B core as a carrier protein for vaccination research

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Abstract The hepatitis B core (HBc) protein has been used successfully in numerous experiments as a carrier for heterologous peptides. Folding and capsid formation of the chimeric proteins is not always achieved easily. In silico analyses were performed to provide further comprehension of the feasibility for predicting successful capsid formation. In contrast to previous work, we show that common in silico predictions do not ensure assembly into particles. We included new considerations regarding capsid formation of HBc fusion proteins. Not only the primary sequence and the length of the inserts seem important, also the rigidity, the distance between the N and the C-terminus and the presence of cysteines, which could form disulphide bonds, could influence proper capsid formation. Furthermore, new conformational insights were formulated when linkers were added to create extra flexibility of the chimeric particles. Different hypotheses were suggested to clarify the obtained results. To this extent, the addition of glycine-rich linkers could lower high rigidity of the insert, removal of the strain of the core protein or ease interaction between the HBc and the insert. Finally, we observed specific changes in capsid formation properties when longer linkers were used. These findings have not been reported before in this and other virus-like particle carriers. In this study, we also propose a new high-yield purification protocol for fusion proteins to be used in vaccination experiments with the carrier protein or in comparative studies of particulate or non-particulate HBc fusion proteins.

Keywords Hepatitis B core · Folding · Purification · Carrier

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Abbreviations

BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
CTL	Cytotoxic T lymphocyte
ECF	East Coast fever
GFP	Green fluorescent protein
HBc	Hepatitis B core
HBe	Hepatitis B e antigen
MIR	Major immunodominant region
NBT	Nitroblue tetrazolium
PIM	Polymorphic immunodominant molecule
PPP	PIM polypeptide
PVDF	Polyvinylidene fluoride

Introduction

The Hepatitis B virus core (HBc) particle is an icosahedral nucleocapsid consisting of 183 aa (amino acid) monomers. The C-terminal region (39 aa) is arginine rich (Gallina et al. 1989). The HBc self-assembles into capsids through the N-terminal (~144 aa) domain. The HBc assembles into capsids of two sizes, with triangulation numbers of $T = 3$ (~280 Å) and $T = 4$ (~310 Å) consisting of 90 and 120 dimers, respectively (Crowther et al. 1994; Wingfield et al. 1995). Each dimer forms a T-shaped structure, with the stem forming a dimer interface that protrudes from the virus as a spike. The cross pieces are responsible for the formation of the contiguous shell (Bottcher et al. 1997; Conway et al. 1997). The dimer assembly is partly driven by two intermolecular disulphide bridges between Cys48 and Cys48′, and Cys 61 and Cys 61′ (Nassal et al. 1992).

Several reports have described the expression of HBc in heterologous hosts, including bacteria (*Escherichia coli*; Pasek et al. 1979), yeast (*Saccharomyces cerevisiae*; Miyanohara et al. 1986), mammalian cells (rat fibroblast cell lines; Roossinck et al. 1986), insect cells (*Spodoptera frugiperda* SF9 cell line; Lanford and Notvall 1990) or frog oocytes (*Xenopus*; Standring et al. 1988). Furthermore, when expressed in bacteria, the HBc particles are structurally similar to native particles (Birnbaum and Nassal 1990; Hatton et al. 1992). When expressed in an attenuated *Salmonella* strain, the HBc has been shown to work as a potential oral vaccine carrier (Schodel et al. 1994).

Because the HBc is repeated up to 240-fold in an intact virus particle, it can elicit a strong immunogenic response (reviewed in Pumpens and Grens 2002; Pumpens et al. 2008). HBV infected individuals mount a high titered anti-HBc antibody response, as can be expected from a highly repetitive antigen (Schodel et al. 1994). The core protein can also directly activate B cells; thereby, acting as a T cell-dependent antigen (Milich and McLachlan 1986). In addition, the HBc as a protein antigen elicits T helper and CTL cell responses (Milich et al. 1987; Schodel et al. 1996).

As shown in numerous studies, the HBc can be used as an ideal carrier for foreign B cell and/or T cell epitopes in vaccination experiments (reviewed in Milich et al. 1995; Pumpens et al. 1995; Schodel et al. 1996; Ulrich et al. 1998; Pumpens and Grens 2001). Insertions have been made at both the N-terminus (Clarke et al. 1987) and C-terminus (Borisova et al. 1989) as well as in the major immunodominant region (MIR) (Schodel et al. 1990), the latter being the central region of a monomer encoding for the spike (between amino acids 70–95 which is in accordance with the position of the epitope c1/e1, located on the tip of the spike; Salfeld et al. 1989). The capacity of each of these insertion sites seems to be different and also the length range of possible insertions differs from site to site (Pumpens and Grens 2001). The highest immunoreactive effectiveness could be raised through insertion into the MIR domain.

As mentioned above, the maximum size of the insert may be limited, depending on the nature of the insert and/or the position in the HBc. The longest inserts thus far introduced are green fluorescent protein (GFP–238 aa) (Kratz et al. 1999) and the immunoprotective region of the Hantavirus nucleocapsid (120 aa) (Koletzki et al. 1999). For the latter, when tested in all insertion sites, only the MIR domain could successfully be used as an insertion site. Moreover, Schodel et al. (1992) reported the correlation between the position of heterologous epitopes in the core protein and the immunogenicity. When compared with the N and C-terminal positions, the internally inserted fusion protein induced highest amount of immune responses. Other research groups proved that MIR-inserted epitopes can even induce an antigenicity of up to 5–10 times higher than the N and C-terminal insertions (Brown et al. 1991; Borisova et al. 1996; Fehr et al. 1998). In addition, antigen-specific B cell epitopes expressed on complete recombinant particles elicit significantly higher antibody titers than when the proteins are used in their native form (Schodel et al. 1992).

The MIR as insertion site for the HBc as a carrier is most beneficial, since the immunogenic features of the HBc are carried over to the inserted polypeptides. This can be explained by the abrogation mentioned above of the humoral response directed to the core being transferred to the inserted epitopes. Moreover, it has been shown that the high immunogenicity of HBc chimeric particles allows the complete exclusion of adjuvants (Stahl and Murray 1989; Brown et al. 1991). In addition, T cell independence can be transferred to the foreign epitopes presented on recombinant core particles (Fehr et al. 1998). In some cases, it has been reported that even the CTL response is increased by the use of the HBc as a carrier in vaccination experiments (Chen and Li 2006).

It can be stated that the core protein of hepatitis B is very flexible in many ways by conserving its structural stability after integration of peptides of different length. Significant immunity could be induced against HBV itself (Chen et al. 2002), and when used as a carrier also against influenza A (Neirynck et al. 1999), hepatitis C (Chen and Li 2006), foot and mouth disease virus (FMDV; Clarke et al. 1987) and *Plasmodium* spp. (Schodel et al. 1997; Milich et al. 2001) through inclusion of their respective protective antigens. The core protein has been extensively used in these and other vaccination strategies because it has several advantages as a carrier for foreign epitopes. HBc fusion proteins improve the immunogenicity of intrinsically low immunogenic peptides presented on their surface (Clarke et al. 1987; Francis et al. 1990).

However, many questions remain concerning the role of the insert size and composition in the ability of HBc particles to present these immunogenic inserts. In our study, we used parts of the polymorphic immunodominant molecule (PIM) protein in constructs inserted in the MIR domain. PIM is a dominant antigen (Toye et al. 1995) and one of the surface antigens of *Theileria parva*. This parasite that causes East Coast fever, a tickborne cattle disease is related to *Plasmodium* spp. and causes high economic losses in large areas of eastern and southern Africa (168 million USD per year; Mukhebi et al. 1992). The high immunoreactivity of this protein has been illustrated (Geysen et al. 2004).

We tested several constructs using linkers to improve particle assembly by means of transmission electron microscopy analysis. We found that linker size has a strong effect on the ability to assemble into virus particles, and on the particle size. In addition, particle assembly prediction has been compared with the earlier published results. We also report on a new and efficient protocol to purify the fusion proteins.

Materials and methods

Construction of expression vectors

The construction of the pBAD/B-HBc vector has been previously described (Broos et al. 2008). In total, 14 gene sequences were inserted in the pBAD/B-HBc vector (Invitrogen), all derived from the *pim* gene. Table 1 shows all different inserts with their respective linkers.

The gene parts encoding for PIMpp (PIM polypeptide) 1 and PIMpp2 were obtained from the *T. parva* Muguga strain (Baylis et al. 1993), the one encoding for PIMpp3 from the Katete B2 strain and the remainder of the inserts (PIMpp4–PIMpp14) from the Katete4 strain (Geysen et al. 1999).

Linkers were added by PCR to the constructs PIMpp1–PIMpp8 similar to those used by Kratz et al. (1999) [$(G)_4S(G)_4$ flanking each side of the insert] since they have proven successful in facilitating the insertion of a relatively long peptide (GFP; 238 aa). Because some of the produced chimeric particles did not particulate after expression (PIMpp4, PIMpp5 and PIMpp8), longer linkers were used in these cases, encoding for $(G)_{10}S(G)_4$ and $(G)_{15}S(G)_4$ or $(G)_4S(G)_{10}$ and $(G)_4S(G)_{15}$ on the N-terminal or on the C-terminal side of the insert, respectively. These constructs encode for polypeptides PIMpp9–PIMpp14. All polypeptides were inserted between amino acids 78 and 79 of the HBc.

RNA of the three different *T. parva* strains was purified and cDNA was prepared before PCR amplification of all *pim* genes used in this study (first-strand cDNA synthesis, Invitrogen). Consequently, primers (underlined sequences in Table 1) were designed to amplify the specific gene parts of the *pim* genes. To the 5′ termini of the primers, extensions were added that contained the sequence for the different linkers and the used restriction sites (*Hpa*I and *Nhe*I). Then, *ppp*1–*ppp*14 were amplified by PCR. All obtained PCR products were ligated in the pGEM-T vector (Promega) and transformed in JM109 *E. coli* cells. After plasmid purification, the pBAD/B-HBc vector and the pGEM-T-PIMpp1-14 vectors were digested with *Hpa*I and *Nhe*I. Afterwards, *ppp*1-14 were added to the digested pBAD/B-HBc vector and the mix was ligated with T4-DNA ligase (New England Biolabs). Finally, the ligation products were transformed into MC1061 *E. coli* cells.

Expression and purification of recombinant particles

Of the transformed MC1061 *E. coli* cells containing pBAD/B-HBc-PIMpp1-14 vectors, 50 ml of a saturated culture of each colony expressing a different HBc-PIMpp protein was added to 450 ml Luria–Bertani medium (Sambrook and Russel 2001) supplemented with carbenicillin (100 μg/ml). These cultures were inoculated in an air shaker (180 rpm) at 37°C for approximately 3 h until an OD_{600} of 0.8 was reached. After induction with L-arabinose (final concentration 0.02% w/v), the cells were harvested and centrifuged at 7,000*g* for 5 min. The obtained pellets were re-suspended into a wash buffer (50 mM Tris/HCl, 50 mM NaCl, pH 8) and centrifuged under the same conditions. Washed pellets were thereafter re-suspended to a final volume of 20 ml in the wash buffer (per litre *E. coli* culture) and lysed using a French press (10,000 psi). Lysed cells were centrifuged at 10,000*g* for 30 min and the supernatant was purified.

This protocol was carried out at 4°C; 33 μl DNaseI (Fermentas) and 20 μl $MgCl_2$ (2 M) were added to the supernatant and incubated for 1.5 h. These samples were centrifuged at 10,000*g* for 30 min and the supernatant was applied on a diethylaminoethyl (DEAE) Sephacel column (10 ml; GE Healthcare Life Sciences) and the flow through was collected. 50 mM Tris/HCl, 40% ammonium sulphate, pH 8 was added to obtain a final concentration of 20% w/v ammonium sulphate. This mixture was incubated for 3 h and then centrifuged for 30 min at 10,000*g*. Subsequently, the pellet was re-suspended in 25 ml wash buffer supplemented with 0.01% Triton X-100 (Sigma-Aldrich Co.) and incubated overnight. The mixture was then centrifuged for 30 min at 10,000*g* and the supernatant was subjected to another ammonium sulphate precipitation, this time with a final concentration of 15% ammonium sulphate, incubated for three subsequent hours and centrifuged for 30 min at 10,000*g*. The pellet was re-suspended in 2 ml wash buffer

Table 1 Protein sequences of the DNA regions amplified by PCR (primers: underlined sequences)

Name insert	Sequence	P/ N-P	Size (nm)	Sol./ Insol.
PIMpp1	vnled*GGGGSGGGGS*TGSSDVTQVDTESNDTSSSSETSQQGKPQPDQPQDQPDQHQQPTQGDTSGQQGPDTPQPIQEPSGPVQPDQTGQGPVEPVDQQQQ PTQGDTS*GGGGSGGGG*pas	P	32 ± 2	S 0.85
PIMpp2	vnled*GGGGSGGGGSGGG*QQPGPDTPDQPVYQQQPVQQPSGQQQQPQPRPQPQPDQPVDQQQEPPTPEDQPSGPDSPDQPDQHHQPTPAAQPQTQPQPEPQPE PQPEPQPEPQPEPVQEPPEQTPEHTPSKD*GGGGSGGGG*pas	P	31 ± 1	S 0.95
PIMpp3	vnled*GGGGSGGGG*PQPEPQPEPQPEPVQEQTPETPAQQEQPQTPDDQTPEQKPDQPVQEPPEQTPEHTPSKDDASGEVPVKPSEGHMTGAAADGSGQPPD KKTDDDSKGKDGSKSGSGTPSKDKKDSKHTPFHGC*GGGGSGGGG*pas	P	32 ± 2	S 0.67
PIMpp4	vnled*GGGGSGGGG*MGSDLNPLDPLAQQSGGGSNNNEGNNNDSTGSSDVTQVDTESNDTSSSSETSQQGKPQPDQPQDQPDQHQQPTQGDTSGQQGQQP QDQPVQEQDGQDSQGTPEQTPDQSGQQPVQQPSGQQQQQPQPEPQPEPQPDQPQDQPDQHQQ*GGGGSGGGG*pas	N-P	n/a	S 0.71
PIMpp5	vnled*GGGGSGGGG*QQQQPQPEPQPEPVQEQTPETPAQQEQPQTPDDQTPEQKPDQPVQEPPEQTPEHTPSKDDASGEVPVKPSEGHMTGAAADGS GQPPDKKTDDDSKGKDGSKSGSGTPSKDKKDSKHTPFHGCGQFFTNTHKVTVIFHWWLCEKPWQ*GGGGSGGGG*pas	N-P	n/a	I 0.56
PIMpp6	vnled*GGGGSGGGGS*DLNPLDPLAQQSGGGSNNNEGNNNDSTGSSDVTQVDTESNDTSSSSETSQQGKPQPDQPQDQPDQHQQPTQGDTSGQQGQQPQD QPVQEQDGQDSQGTPEQTPD*GGGGSGGGG*pas	P	31 ± 1	S 0.72
PIMpp7	vnled*GGGGSGGGGQ*DGQDSQGTPEQTPDQSGQQPVQQPSGQQQQPQPEPQPEPQPEPVQEQTPETPAQQEQPQTPDDQTPEQKPDQPVQEPPEQTPEHTP SKDDASGEVPVKPSEGH*GGGGSGGGG*pas	P	30 ± 2	S 0.90
PIMpp8	vnled*GGGGSGGGG*KDDASGEVPVKPSEGHMTGAAADGSGQPPDKKTDDDSKGKDGSKSGSGTPSKDKKDSKHTPFHGCGQFFTNTHKVTVIFHWWLC EKPWQ*GGGGSGGGG*pas	N-P	n/a	S 0.67
PIMpp9	vnled*GGGGGGGGGGSGGG*MGSDLNPLDPLAQQSGGGSNNNEGNNNDSTGSSDVTQVDTESNDTSSSSETSQQGKPQPDQPQDQPDQHQQPTQGDTS GQQGQQPQDQPVQEQDGQDSQGTPEQTPDQSGQQPVQQPSGQQQQQPQPEPQPEPQPDQPQDQPDQHQQ*GGGGSGGGGGGGGGG*pas	P	27 ± 2	S 0.78
PIMpp10	vnled*GGGGGGGGGGSGGG*QQQQPQPEPQPEPQPEPVQEQTPETPAQQEQPQTPDDQTPEQKPDQPVQEPPEQTPEHTPSKDDASGEVPVKPSEGHMTG AAADGSGQPPDKKTDDDSKGKDGSKSGSGTPSKDKKDSKHTPFHGCGQFFTNTHKVTVIFHWWLCEKPWQ*GGGGSGGGGGGGGG*pas	P	22 ± 2	I 0.58
PIMpp11	vnled*GGGGGGGGGGSGGGK*DDASGEVPVKPSEGHMTGAAADGSGQPPDKKTDDDSKGKDGSKSGSGTPSKDKKDSKHTPFHGCGQFFTNTHKVTVI FHWWLCEKPWQ*GGGSGGGGGGGGG*pas	P	22 ± 2	S 0.73
PIMpp12	vnled*GGGGGGGGGGGGGGGSGGG*MGSDLNPLDPLAQQSGGGSNNNEGNNNDSTGSSDVTQVDTESNDTSSSSETSQQGKPQPDQPQDQPDQHQQPT QGDTSGQQGQQPQDQPVQEQDGQDSQGTPEQTPDQSGQQPVQQPSGQQQQQPQPEPQPEPQPDQPQDQPDQHQQ*GGGGSGGGGGGGGGGGGGG*pas	P	21 ± 2	S 0.68
PIMpp13	vnled*GGGGGGGGGGGGGGGSGGG*QQQQPQPEPQPEPQPEPVQEQTPETPAQQEQPQTPDDQTPEQKPDQPVQEPPEQTPEHTPSKDDASGEVPVKPSE GHMTGAAADGSGQPPDKKTDDDSKGKDGSKSGSGTPSKDKKDSKHTPFHGCGQFFTNTHKVTVIFHWWLCEKPWQ*GGGSGGGGGGGGGGGGGG* pas	P	23 ± 2	I 0.54
PIMpp14	vnled*GGGGGGGGGGGGGGGSGGG*KDDASGEVPVKPSEGHMTGAAADGSGQPPDKKTDDDSKGKDGSKSGSGTPSKDKKDSKHTPFHGCGQFFTNT HKVTVIFHWWLCEKPWQ*GGGSGGGGGGGGGGGGGG*pas	P	24 ± 3	S 0.63

Gene sequences were inserted with the *Hpa*I and *Nhe*I restriction enzymes, recognising sequences GTTAAC and GCTAGC respectively, corresponding to the bold sequences at N and C-terminal ends

The small letters correspond with the HBc sequence, the capital letters with the insertions

The third column represents the successful or unsuccessful folding of the fusion protein (*P* particulate; *N-P* non-particulate)

The next column shows the size of each chimeric particle according to the measurement on electron microscopical pictures

In the last column the propensity of the inserts to be soluble (S) or insoluble (I) upon overexpression in *E. coli* is shown Linkers are highlighted in Italic

Sequences highlighted in the same colour are identical

and dialysed overnight using 5 l wash buffer. After dialysis, the remainder was cleared by centrifugation (30 min at 10,000*g*). Finally, the supernatant was loaded on a gel filtration column (Sephacryl S-300, GE Healthcare Life Sciences) and the protein fractions, corresponding to the void, collected.

Analytical methods

The different protein purification steps were checked on sodium dodecyl sulphate-polyacrylamide (SDS-PAA) gels (Laemmli 1970) stained with Coomassie brilliant blue. Western blot analysis was performed after transfer of the proteins to an Immobilon-P polyvinylidene difluoride (PVDF) transfer membrane (Millipore). Afterwards, proteins were detected using a primary polyclonal rabbit anti-HBc antibody (DakoCytomation, Carpinteria CA, USA) and an alkaline phosphatase labelled goat anti-rabbit antibody (Sigma-Aldrich Co.) as a secondary antibody for the identification of the primary antibodies. Regarding the chimeric particles, monoclonal mouse anti-PIM antibodies (IL-S36.5) were used, followed by incubation with an alkaline phosphatase labelled rabbit anti-mouse antibody (Sigma-Aldrich Co.). Detection was carried out with the 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium colouring agents. Protein quantities were measured by the Bradford (1976) assay and the Schaffner and Weissman (1973) assay using BSA as a standard.

Microscopy

Particle assembly was ascertained by observation using transmission electron microscopy (Philips CM10, 1989); 3 µl of a 150 µg/ml purified HBc-PIMppn chimeric protein was adsorbed to a Formvar/Carbon support film grid (#FCF200CU Laborimpex). As a negative stain, 1% phosphotungstic acid (pH 8) was used (Palmer and Martin 1988). Diameters of the particles were determined with ImageJ 1.42 software (Abramoff et al. 2004).

Results

A novel high efficiency purification protocol for HBc chimeric particles

HBc chimeric particles have typically been purified using sucrose gradients (Geldmacher et al. 2004). However, this method imposes a limit on the batch sizes. Alternatively, to obtain purified particles, other protocols such as chromatography and precipitation methods have been suggested, but good yields are difficult to obtain, especially when working with different fusion proteins. In this study, we purified all HBc–PIMppn fusion proteins with a novel protocol based on the several steps alternating anion exchange, ammonium sulphate treatment and gel filtration chromatography. A protein purity of 85–90% could be obtained (see Coomassie stained SDS-PAA gel in Fig. 1a). Moreover, a yield of 0.8–2.5 mg pure protein per litre of bacterial culture was acquired within 3 days [measured with the Schaffner and Weissmann (1973) method]. The differences in yield depend on the fusion protein, with particulate proteins giving a higher yield than non-particulate although both can be purified. For the native HBc we obtained approximately 1.8 mg per litre of bacterial culture. In addition, the protocol can be easily up-scaled through more efficient induction of the *E. coli* cells. Application of a bioreactor for induction increased HBc production up to five times (K. Broos, unpublished results). Specific fusion proteins were identified by Western blots (Fig. 1b, c).

Prediction of possible capsid formation and comparison with published data

We tested a series of inserts some of which led to the unsuccessful folding, and therefore added an extra number of specific constructs to our analysis (Table 1).

Their ability to form particles was analysed using electron microscopy. Out of the first eight constructs, three (constructs 4, 5 and 8) did not assemble into symmetric particles. All other fusion proteins assembled properly into particles (Fig. 2).

As stated by Karpenko et al. (2000), assembly of chimeric particles could be predicted by the calculation of the hydrophobicity, β-strand index and volume of the 7–10 last amino acids on the C-terminal side of the inserted polypeptide. We also included all protein fusions discussed in Karpenko et al. (2000) in the analysis of our inserts, as well as the GFP protein in the analysis because Kratz et al. (1999) successfully inserted this protein into the HBc.

Possible particle assembly was predicted in silico with different programmes, i.e. two secondary structure prediction programmes (CHOFAS, Chou and Fasman 1978; GOR4, Garnier et al. 1996) and a hydrophobicity programme (Kyte and Doolittle 1982). For the secondary structure prediction, all insertion protein sequences were analysed and a score was given only if β-strand formation was predicted using the 10 last amino acids of the C-terminus. In case of the CHOFAS algorithm, the score ranged between 1 and 10 (10 if all amino acids are predicted to form β-strands; 0 if none of the amino acids do) whereas the GOR4 algorithm score was dependent on each amino acid crossing the threshold of 0.8, as calculated by the

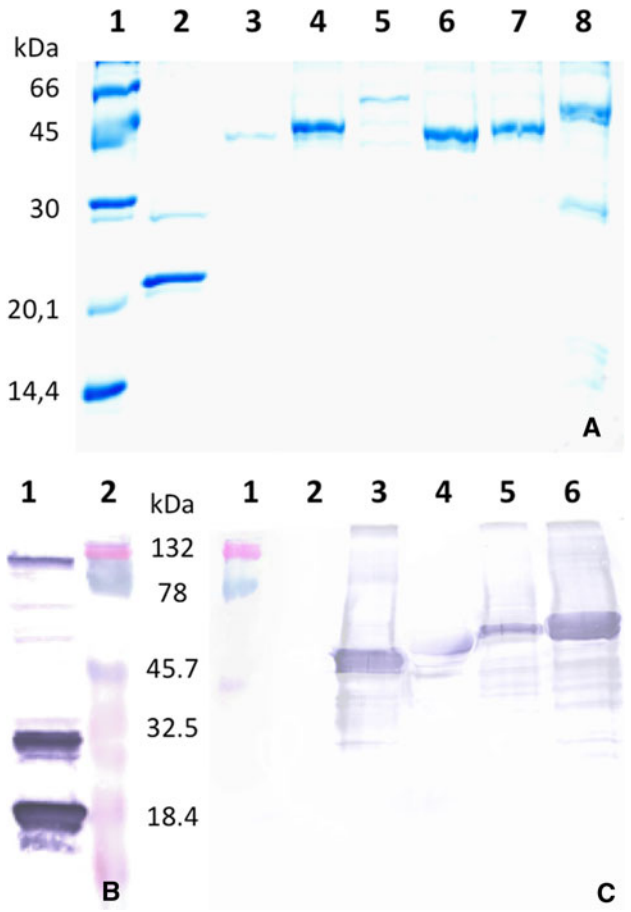

Fig. 1 SDS-PAA gel analysis of the purified fusion proteins (purification protocol: see text). After SDS-PAA, the standard proteins were visualised by Coomassie blue staining (**a**) or blotted onto Immobilon-P PVDF transfer membranes and proteins were detected as described in "Materials and methods" (**b**, **c**). **a** *Lanes 1* low molecular weight marker (Amersham), *2* purified HBc, *3–8* purified HBc–PIMpp1, HBc–PIMpp3, HBc–PIMpp4, HBc–PIMpp6, HBc–PIMpp7 and HBc–PIMpp12, respectively. **b** *Lanes 1* kaleidoscope prestained ladder (Bio-Rad), *2* purified HBc. Antibodies used: primary polyclonal rabbit anti-HBc antibody (DakoCytomation, Carpinteria CA, USA) and secondary alkaline phosphatase labelled goat anti-rabbit antibody (Sigma-Aldrich Co.). **c** *Lanes 1* kaleidoscope prestained ladder (Bio-Rad), *2* purified HBc, *3–5* purified HBc–PIMpp1, HBc–PIMpp3, HBc–PIMpp4, and HBc–PIMpp12, respectively. Antibodies used: primary monoclonal mouse anti-PIM antibodies (IL-S36.5) and secondary an alkaline phosphatase labelled rabbit anti-mouse antibody (Sigma-Aldrich Co.)

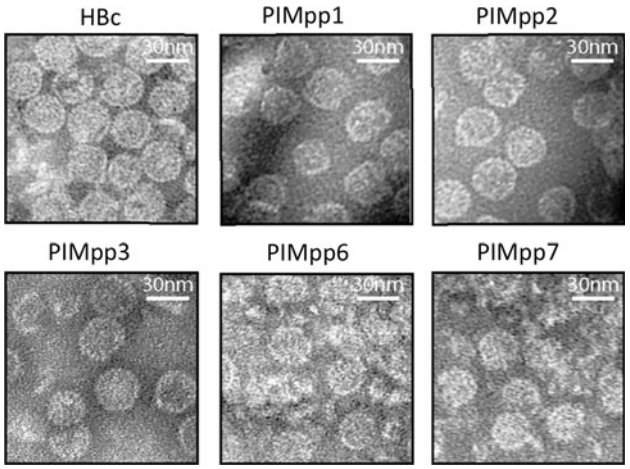

Fig. 2 Electron micrographs of different purified fusion particles and recombinant HBc particles. Above pictures of the fusion proteins, the name of the respective insert is shown. Each picture represents a different protein. *White bar* represents 30 nm as indicated

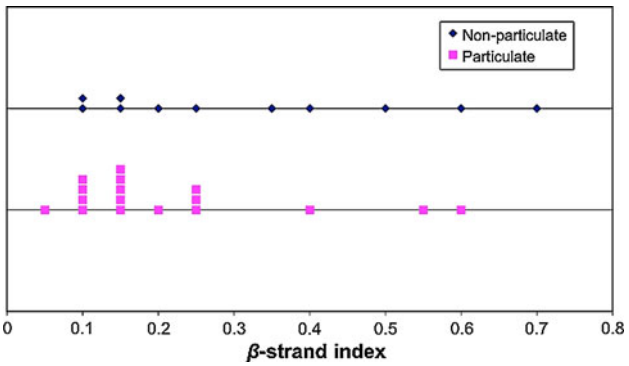

Fig. 3 Comparison of the β-strand indices of the particulate and non-particulate constructs analysed in this study. Indices are based on the two different algorithms as discussed in the text. A tendency to a higher index is noticed with the non-particulate fusion proteins. When compared with the calculations made by Karpenko et al. (2000) our findings seem to be different. When excluding certain indices of chimeric proteins not discussed in the latter article [e.g. new fusion proteins, which are constructed during this study and GFP, published by Kratz et al. (1999)], our results are comparable

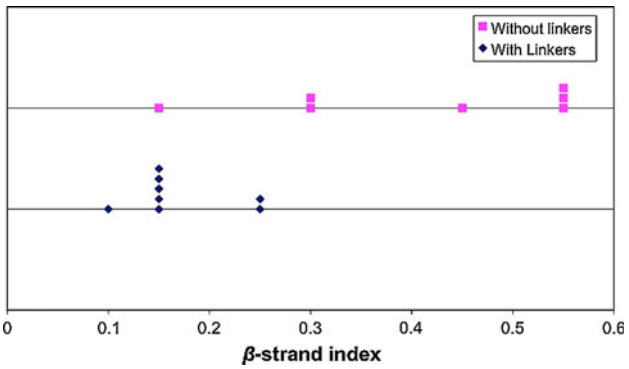

Fig. 4 Comparison of the β-strand indices of the fusion proteins HBc–PIMpp1-8 with the 9 aa linkers and putative constructs of the same inserts, but without these linkers. Indices are based on the two different algorithms, as discussed in the text. This figure shows clearly that the indices of the inserted polypeptides are drastically lowered when linkers are added. Nevertheless, this could not ensure appropriate folding of the fusion proteins

programme itself. If all ten specific amino acids yielded a GOR4 algorithm score above 0.8, a score of 10 was given; if none exceeded 0.8, a score of 0 was given. Finally, the average was calculated by merging both scores. The scores of the hydrophobicity algorithm were used as such.

Figure 3 shows a summary of the calculated β-strand indices of all fusion proteins, divided into two groups: particulate and non-particulate proteins. We also calculated the β-strand indices of the inserts without any linkers (Fig. 4). The addition of these linkers clearly lowers the β-strand index of the last C-terminal amino acids, but does not always guarantee assembly.

We did not consider the volume of the inserts in our analysis, since the linkers contain high numbers of glycine, already being the smallest amino acid. Moreover, analysis of the hydrophobicity of all the fusion proteins mentioned in this study, did not give any correlations. The widely applied Kyte and Doolittle (1982) algorithm was used and regions with values above 0 are hydrophobic in nature. In addition, the propensity of the inserts to be soluble upon overexpression in *E. coli* was predicted (SOLpro, Magnan et al. 2009; Table 1). Testing the particulate and non-particulate proteins with these algorithms resulted in divergent results, but were not in any useful correlation.

To complete the analysis, the proteins were also tested for the presence of known domain structures. To this end, we used the Phyre server (Bennett-Lovsey et al. 2008). None of these peptides showed an *E* value below 35 (estimated precision of containing potential domains was maximally 5%) pointing to the absence of correlation with known domains, which indicates that they are folded intrinsically. This analysis was also performed on the already published inserts some of which contained potential domains (while others did not). Nevertheless, no correlation between particulate and non-particulate constructs could be observed.

Longer linkers result in different particles

Because three of our constructs did not allow assembly into particles, we argued that the insertions might have been too rigid. We, therefore, increased the length of the linkers to 15 and 20 amino acids (HBc–PIMpp9–14; see Table 1). These constructs were expressed and purified using the same protocol and analysed by electron microscopy. We found that these chimeric proteins did assemble into symmetrical particles (Fig. 5). Surprisingly, they were all

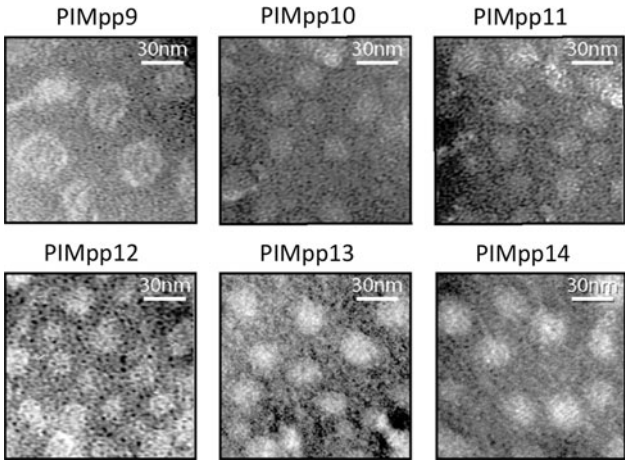

Fig. 5 Electron micrographs of different purified fusion particles. Above pictures of the fusion proteins, the name of the respective insert is shown. Each picture represents a different protein. *White bar* represents 30 nm as indicated

smaller than the native HBc proteins: the diameter of the native HBc and the HBc–PIMppn fusion particles with 9 aa linkers that did assemble was approximately 31 nm and is, therefore, larger than that of the fusion proteins with the larger linkers ranging from 23 to 27 nm (Table 1).

Discussion

The HBc has a number of interesting properties making it an ideal vaccine carrier for foreign B cell and T cell epitopes. To gain more insight into its carrier potential and efficiency, we engineered different constructs and expressed them in *E. coli*. In addition, we successfully developed a general purification protocol and subsequently analysed the effect of the different construct formats on particle formation.

Purifications of proteins are often very time consuming and laborious to implement, requiring adapted protocols for different proteins. In this article, we describe a novel high-yield purification technique applicable to particulate and non-particulate HBc fusion proteins.

The immunodominant region of the HBc has been a useful insertion site for HBc fusion proteins. However, in many cases, the HBc fusions do not form particles (Karpenko et al. 2000; Zhang et al. 2007; own observations), which reduce the immunogenicity and antigenicity of the HBc (Milich et al. 1988). Various hypotheses might explain the failure of some chimeric/fusion particles assembly: (1) very often, the length of the insert appears to be the bottleneck for proper assembly. Until recently, the maximum length of insertions was 120 amino acids (Koletzki et al. 1999), while Kratz et al. (1999) could successfully insert the GFP protein (238 aa) in the HBc, most likely due to the stability of the entire GFP. Successful assembly is often difficult to obtain in other cases (Makeeva et al. 1995; Pumpens and Grens 2001), probably due to the fact that most of the other tested constructs contain partial protein inserts instead of entire proteins. (2) The primary sequence seems to be an important aspect for an optimal folding of the fusion proteins. The potential to obtain efficient assembly has been attributed to β-sheet forming properties, volume and hydrophobicity of the amino acids of the insert (Karpenko et al. 2000). According to our analysis, this hypothesis appears to be inaccurate.

We are convinced that three other explanations are also appropriate. (3) First, the distance between the N and the C-terminus of the insert in its native conformation could disrupt the capsid formation. The measured distance between the two ends of the MIR is approximately 10 Å (measured with PyMOL, DeLano Scientific LLC). It is very unlikely that the core protein is flexible enough to successfully assemble when the polypeptide folds in such a way that both termini are much further away from each other. This is very well illustrated in the case of GFP being a relatively long protein, but the termini lie very close to each other ($\approx$18 Å) due to its barrel conformation when folded natively (Ormo et al. 1996). (4) Second, capsid formation can take place in the absence of disulphide bonds; however, the presence of a cysteine in an insert might interfere with possible folding of the HBc. This has been seen in the HBe antigen, which does not form particles (Nassal and Rieger 1993; Wasenauer et al. 1993). Consequently, one could envision difficulties in proper capsid formation of fusion proteins in the presence of cysteine residues in the inserted polypeptides, especially when considering that multiple cysteine residues are present in the HBc (Nassal et al. 1992). These could also bring the particle in imbalance by forming a bridge with other cysteines in the inserts. Although there is a tendency of higher numbers of cysteines in the non-particulate fusion proteins, we believe that prediction is impossible if folding characteristics are not known as the interaction between certain cysteines depends on the conformation of the protein. (5) Third, not only flexibility of the core, but also the flexibility of the inserts could be an important factor in shell assembly of the HBc fusion proteins. However, it is also very unlikely that capsid formation will take place when the spacial volume of the insert is too high to fit between the amino acids of the MIR. This suggestion is supported by the observation that deletion of certain amino acids of the MIR can facilitate chimeric particle folding (Zhang et al. 2007).

In this study, we also demonstrate that flexible glycine linkers have an effect on proper insertion of polypeptides. Although we showed that these should improve the folding of fusion proteins when analysed with poor in silico

prediction parameters, not all constructed insertions formed particles properly.

Fusion proteins that led to non-particulate proteins with 9 aa linkers did produce particles when the linker length was increased to 15 and 20 aa. However, the particle size decreased from 31 nm (wild-type HBc) to 21 nm (PIMpp12). Changes in diameter of the particles have been previously reported elsewhere; they tend to be larger in case of insertions into the core (Borisova et al. 1993; Kratz et al. 1999) whereas deletion at the C-terminus of the HBc made them smaller in size (Borisova et al. 1993).

Why fusion proteins form smaller particles when longer linkers (15–20 aa) are applied could be the result of the fact that with the 9 aa linkers, the termini lie too far apart and the high rigidity of the insert hinders removal of the strain in the HBc insert interaction (Fig. 6b). 15 aa linkers lower the strain even more and the presence of these longer linkers forces the HBc monomer MIR to undergo some conformational changes to create dimers thereby forming smaller particles as is seen using electron microscopy.

Another reason for this perturbation could be the possible interaction of the inserts with the core itself facilitated by increased flexibility of the longer linkers. This interaction could also change the conformation of the HBc itself. This finding is supported by the observation that longer linkers of 20 aa also result in smaller particles. In this case, the longer the linkers, the more possibilities this creates for flexible interaction.

A third reason for this reduction in size could be explained by the fact that the strain of the core protein is lowered (Fig. 6c). Deletions in the MIR would give rise to similar results. However, no particle size differences in deletion variants in the MIR of the HBc have been reported so far (Preikschat et al. 1999).

After analysis of our fusion proteins and their properties to assemble into proper particles, we can conclude a priori that in silico analysis does not increase the success of this assembly. Thus, whether new chimeric proteins will assemble into shells or not should be tested by trial and error and confirmed by electron microscopy. Using longer linkers shell assembly of HBc fusion proteins is facilitated, but the composed particles are smaller than recombinant HBc particles.

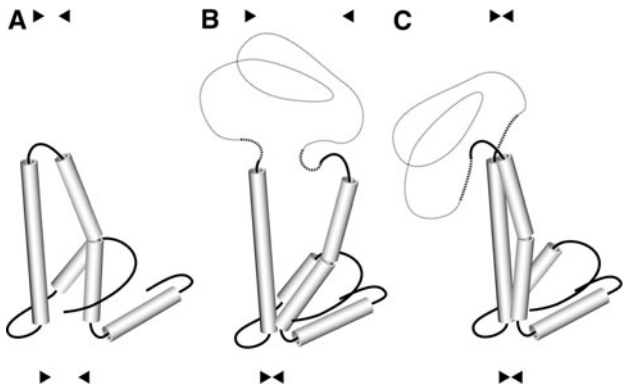

Fig. 6 Native and alternative putative folding of the HBc monomer. **a** The folding pattern of a native hepatitis B core monomer. **b** Flexibility of the HBc monomer for insertions is augmented by the use of linkers, but changes the conformation due to the compulsion of the monomer to fold. **c** Conformational change of the HBc monomer due to the reduction of the strain in the MIR. In both alternative cases (**b**) and (**c**), the C-terminal α-helix and amino acids move closer to the rest of the protein. Because the C-terminal region provides most of the interdimer interactions (Wynne et al. 1999), this type of closer packaging would make the complete shells smaller

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