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Molecular characterization of recombinant Hepatitis B surface antigen from Chinese hamster ovary and *Hansenula polymorpha* cells by high-performance size exclusion chromatography and multi-angle laser light scattering

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Abstract

The molecular weight and size of recombinant Hepatitis B surface antigen (HBsAg) derived from Chinese hamster ovary (CHO) and the *Hansenula polymorph* have been characterized by high-performance size exclusion chromatography with multi-angle laser light scattering (HPSEC-MALLS). The average molecular weight of CHO-derived HBsAg particle (CHO-rHBsAg) (4921 kDa) was higher than that of *H. polymorpha* yeast strain (Hans-rHBsAg) (3010 kDa). The size of CHO-rHBsAg (22.1 nm) is nearly the same as that of native HBsAg compared to 18.1 nm for Hans-rHBsAg. The average monomer numbers were found to be 155 for CHO-rHBsAg and 86 for Hans-rHBsAg, respectively. The data obtained support the assumption that the higher immunogenicity of CHO-derived HBsAg is related to its more favorable macromolecular assembly structure.

Keywords: Hepatitis B surface antigen (HBsAg); Molecular weight; Molecular size; Distribution; HPSEC-MALLS

1. Introduction

Hepatitis B virus (HBV) is the causative agent of a worldwide disease that affects an estimated 400 million people, especially in Africa and East Asia [1]. Since no specific remedy against HBV has yet been developed, inoculation with Hepatitis B vaccine is the most effective means of preventing HBV infection and its consequences. The first generation vaccine was the Hepatitis B surface antigen (HBsAg) derived from human plasma of chronic carriers [2]. HBsAg in the plasma is present in the form of 42 nm HBV infectious viral particles as well as 22 nm non-infectious particles that are free of DNA and other HBV structural proteins [3]. The highly immunogenic 22 nm HBsAg particles are composed of protein, lipid and carbohydrate moieties [4]. The plasma-derived HBsAg particles have been employed in immunization programs as an effective vaccine for decades. However, its major drawbacks, such as potential health hazard and limitation of supply, have prevented WHO to recommend it for more widespread use [5]. The development of recombinant HBsAg (rHBsAg) has made the possible use of eukaryotic expression hosts such as various yeast strains (*Saccharomyces cerevisiae*, *Pichia pastoris* or *Hansenula polymorpha*) and mammalian cell lines (CHO) [6–9]. The rHBsAgs have the same primary structure of forming self-assembled virus-like particles (VLPs) as their natural counterpart, but are safer and still highly effective as vaccines. However, the immunogenicity of HBsAg derived from different expression systems varies significantly [10]. The CHO-derived HBsAg particle (CHO-rHBsAg) has got a glycosylated structure and the composition is more similar to that of plasma-derived HBsAg comparing yeast-derived HBsAg [11]. Studies regarding the relationship between the immunological properties of the vaccine and its physico-chemical properties, such as the HBsAg composition and structure, play an important role when investigating the functional mechanism of the VLPs in vivo or in vitro [12–14]. It has been reported that the immunogenicity of the 22 nm HBsAg particles assembled from about

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100 monomers was about 1000-fold higher than that of unassembled HBsAg protein [15]. Diminsky et al. [11] compared the composition, structure and immunogenicity differences between HBsAg particles derived from a CHO cell line and a *H. polymorpha* yeast strain (Hans-rHBsAg). The investigation was based on rHBsAg encoded by different gene sequences: Hans-rHBsAg by S and CHO-rHBsAg by pre-S_1, pre-S_2 and S region, which might result in varying immunogenicity. Ishikawa and co-workers [16] compared the molecular weights and particle sizes of two kinds of yeast-derived rHBsAg particles using high-performance size exclusion chromatography (HPSEC) combined with low angle laser light scattering (LALLS). The data obtained showed that the HBsAg particles were size homogeneous, which is contrary to other reports that show a broader size distribution in SEC analysis [16]. So, even if different rHBsAgs are encoded by the same gene sequence, when derived from different expression systems they may have different immunogenicity because of varying molecular weight, molecular size and number of HBsAg monomers in their respective VLPs. However, detailed knowledge of these functional relationship phenomena is still lacking.

HPSEC is a widely used method for molecular weight estimation of biological macromolecules. It is normally based on the measurement of specific elution volumes and the use of calibration standards, however, not only molecular weight and shape but also the possible effect of electrostatic and other interactions with the matrix must be compensated for. This is why this method is not very accurate when absolute molecular weight data are required [17]. Laser light scattering (LLS) provides a direct and absolute measure of molecular weight and structural parameters, calibration standards are not required and it is independent of possible interactions with the matrix. The average molecular weight (M_w), and the corresponding Z-average radius mean square gyration (RMS), i.e., radius of gyration, r_g, are directly obtained by the combination of HPSEC and LLS [18]. Furthermore, the combination of HPSEC and multi-angle laser light scattering (MALLS) makes it possible to analyze size distributions of highly polydisperse samples and to obtain both differential and cumulative distributions of the molecular weight and the mean square radius. It has been widely applied for the determination of protein molecular weights, molecular size and their distributions [19–23].

In the current investigation the molecular weight (M_w), particle size and size distributions, the monomer number of CHO-rHBsAg and Hans-rHBsAg, encoded by the same S sequences were compared by means of HPSEC combined with multi-angle laser light scattering, refractive index and UV absorbance, for the purpose of studying the relationship between the immunogenicity and the particle assembly structure.

2. Experimental

2.1. Chemicals and equipment

All chemicals were analytical grade reagents. Human serum albumin (HSA) and bovine serum albumin (BSA) monomer were obtained from Sigma (USA). The chromatography systems, ÄKTAexplorer 100 and BioPilot, and all the gels for chromatography were purchased from GE Healthcare Bio-Sciences, Uppsala, Sweden. All solutions were made using Mili-Q grade water (Millipore, USA).

2.2. Preparation of purified recombinant HBsAg sample for HPSEC

Chinese hamster ovary (CHO) cells, containing the coding region for the S protein of HBsAg, were used for the production of the CHO-rHBsAg particles. CHO cell cultures were grown in roller bottles containing Dulbecco's modified Eagle's medium (DMEM) and 5% (v/v) fetal calf serum. Every two days after the cells reached confluence, the cell culture medium was harvested and the same volume of fresh medium was added to each roller bottle. The collected culture media were centrifuged at $6000 \times g$ for 30 min to remove cell debris followed by concentration by precipitation or ultrafiltration. The purified HBsAg particles were purified using a combination of hydrophobic interaction chromatography, anion exchange chromatography, concentration by ultrafiltration and gel filtration chromatography using a previously published procedure [24]. The purified CHO-rHBsAg was concentrated prior to HPLC by ultrafiltration using a Microcon YM-10 tube (Millipore, USA). The standard CHO-rHBsAg was a gift from North China Pharmaceutical Group Corporation (NCPC, Shijiazhuang, China).

Fermentation of the methylotrophic *H. polymorpha* yeast cells, containing the coding region for the S protein of HBsAg, was used for the production of the Hans-rHBsAg particles. After the methanol induction the cells were harvested by centrifugation followed by disruption using a high-pressure homogenizer. The cell debris was removed by centrifugation, repeated twice. Finally, a combination of anion exchange chromatography, hydrophobic interaction chromatography and size exclusion chromatography was applied to obtain the purified Hans-rHBsAg.

The purified CHO-rHBsAg and Hans-rHBsAg samples were concentrated by ultrafiltration using an YM-10 tube, and then subjected to treatment with Tween 80 (1:50, v/v) and DTT (0.1 mol/l) at the ratio of 1:1 (v/v) for at least 2 min at room temperature in order to increase the chromatographic resolution [25]. Samples of CHO-rHBsAg and Hans-rHBsAg, with purities higher than 95%, were analyzed by SDS–PAGE under reducing conditions. The protein concentration was measured using a modified Bradford's method [26].

2.3. High-performance size exclusion chromatography with multi-angle laser light scattering (HPSEC-MALLS)

A high-performance liquid chromatographic system (Agilent 1100, USA) with a variable wavelength UV detector equipped with a TSK G5000PWxl (300 mm × 7.8 mm) column and a PWxl Guard column was connected to a multi-angle light scattering detector (DAWN EOS, $\lambda = 690$ nm, Wyatt Technology Corp., USA) and a refractive index detector (OPTILAB DSP, Wyatt Technology Corp., USA). The eluent was monitored with the three detectors connected in series in the sequence: UV, MALLS and RI. The mobile phase was 50 mM phosphate-buffer

containing 0.05% NaN_3, pH 6.8. The analysis was carried out at a flow rate of 0.5 ml/min at room temperature (ca. 25 °C). The UV and RI detectors were calibrated using sodium chloride and toluene, respectively. The MALLS detector was normalized using BSA followed the protocols recommended by Wyatt Technology.

An accurate estimation of the refractive index increments (dn/dc) value is very important for the calculation of the correct molecular weight. A large amount of HBsAg at high concentration was required for the determination of the dn/dc using the RI detector. Since HBsAg samples tend to aggregate after having been concentrated (to around 0.6 mg/ml), it is difficult to obtain enough RI signal to determine the dn/dc value accurately. It has been reported that the dn/dc value for protein $(dn/dc)_P$, is constant (≈0.186 ml/g) and almost independent of the amino acid composition [21]. The composition of lipids and carbohydrate moiety in recombinant HBsAg is known [11]. The dn/dc values of lipid $(dn/dc)_L$ and of carbohydrate $(dn/dc)_C$ is 0.134 ml/g and 0.148 ml/g, respectively, were obtained from the literature [16,21]. Therefore, it was regarded possible to estimate the dn/dc value of the recombinant HBsAg using the following equation [21]:

$$\frac{dn}{dc} = \frac{M_P(dn/dc)_P + M_L(dn/dc)_L + M_C(dn/dc)_C}{M_P + M_L + M_C} \quad (1)$$

where M is the molecular weight; dn/dc, the refractive index increments; and the subscripts P, L and C stand for the polypeptide, lipids and carbohydrate components, respectively.

Finally, the molecular weight was obtained using the ASTRA® software (Wyatt Technology, USA).

3. Results and discussion

3.1. Determination of molecular weights and molecular weight distributions of recombinant HBsAgs

HPSEC is a standard method for the separation of biological macromolecules according to size, as well as for the determination of mean molecular weights and molecular weight distributions [27]. HBsAg is a polydisperse protein assembly of very large molecular weight (more than 2000 kDa). It is very difficult to measure its molecular weight and weight distribution accurately using the SEC method since appropriate calibration standards with similar molecular weight and conformation as HBsAg are not available. Thus, it is not possible to estimate the absolute molecular weights by conventional SEC. However, the relative molecular weights can still be qualitatively analyzed by retention time comparisons. The retention times based on UV adsorption measurement of the eluate was 14.9 min for CHO-rHBsAg and 15.6 min for Hans-rHBsAg, respectively (Fig. 1), suggesting a larger molecular weight for the former. In order to obtain an absolute molecular weight of recombinant HBsAg, SEC combined with online light scattering and refractive index detectors was applied. The basic light scattering equation is [19]:

$$\frac{K^*C}{R(\theta)} = \frac{1}{M}\left[1 + \frac{16\pi^2}{3\lambda^2}\langle r_g^2\rangle \sin^2\left(\frac{\theta}{2}\right) + \cdots\right] + 2A_2C \quad (2)$$

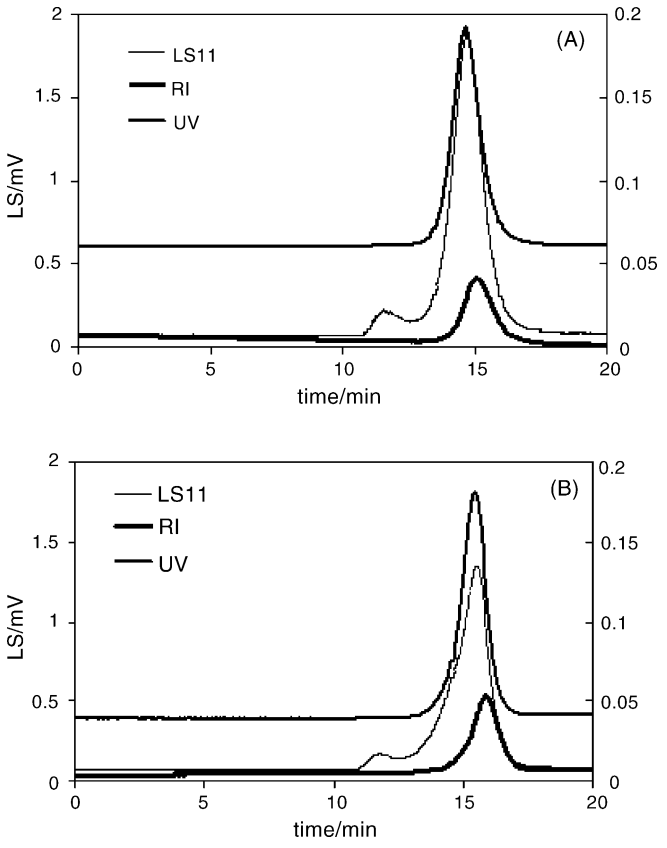

Fig. 1. Profiles of MALLS-HPSEC assay of CHO-rHBsAg (A) and Hans-rHBsAg (B); 100 μl of each sample (ca. 0.2 mg/ml) was injected into a TSK G5000 PWxl column (300 mm × 7.8 mm) G5000PWxl column with 50 mM PB as eluant at a flow rate of 0.5 ml/min.

where K^* is an optical parameter equal to $[4\pi^2n^2(dn/dc)^2]/(\lambda^4N_A)$; C, the solute concentration (mg/ml); $R(\theta)$, the excess intensity of Rayleigh ratio of light scattering at the angle θ; λ, the wavelength; n, the refractive index of the solvent; dn/dc, the refractive index increment; N_A, the Avogadro's number; M, the weight-average molecular weight; (r_g^2), the mean square radius of gyration; A_2, the second virial coefficient, but at the low concentrations usually encountered during column chromatography, the A_2 term is negligible.

For the HBsAg protein containing carbohydrates with $(r_g^2)^{1/2} < \lambda/20$ (or molecular weight $<5 \times 10^7$ g/mol), the term $[16\pi^2(r_g^2)\sin^2(\theta/2)]/(3\lambda^2)$ will generally be negligible at all angles, especially at low angle, and the "three-detector method" can be applied for estimating the molecular and molecular size [21]. The above equation can be simplified to:

$$\frac{K^*C}{R(\theta)} = \frac{1}{M} \quad (3)$$

Thus,

$$R(\theta) = \left[\frac{4\pi^2n^2}{(\lambda_0^4N_A)}\right]\left(\frac{dn}{dc}\right)^2 MC \quad (4)$$

According to Eq. (4), the LS signal ($R(\theta)$) is proportional to $MC(dn/dc)^2$. Since the RI signal is proportional to $C\,dn/dc$ and UV signal is proportional to the concentration of protein

Table 1
Weight-average molecular weights (M_w) for CHO-rHBsAg and Hans-rHBsAg obtained by HPSEC-MALLS

Batch	M_w of CHO-rHBsAg (kDa)	M_w of Hans-rHBsAg (kDa)
1	4889	3004
2	5013	3047
3	4780	2967
4	5001	3023
Average	4921	3010
Derivation (%)	2.8	2.1

moiety, the ratios LS/RI and RI/UV were regarded constant. The narrow elution peaks of CHO-rHBsAg (Fig. 1A) and Hans-rHBsAg (Fig. 1B) suggest that the composition of the HBsAg particles with respect to protein, lipid and carbohydrate is homogeneous. It can be concluded that the refractive index increments (dn/dc) of Hans-rHBsAg and CHO-rHBsAg are nearly constant, when calculated using Eq. (1), where 0.164 ml/g dn/dc for Hans-rHBsAg was obtained from the composition of 59.6% protein and 40.4% lipid [11]. The composition of CHO-rHBsAg expressed by the S sequences was assumed to be the same as that expressed by the whole sequence, containing 59.5% protein, 38.4% lipids and 2.1% carbohydrate [11]. So, a value of 0.165 ml/g dn/dc for CHO-rHBsAg was obtained using Eq. (1). Using the dn/dc values and the ASTRA® software in Eq. (4), the weight-average molecular weights (M_w) of CHO-rHBsAg and Hans-rHBsAg were calculated to be 4921 kDa ($n=4$, derivation = ± 2.8%) and 3010 kDa ($n=4$, derivation = ± 2.1%), respectively (Table 1). Thus, the average molecular weight of the CHO-rHBsAg VLP is significantly larger than that of the Hans-rHBsAg VLP. This indicates that recombinant HBsAgs, even when encoded by the same DNA sequences but expressed using different expression systems, has got varying molecular characteristics, and, as a consequence, possibly different chemical, physical and immunological properties.

The molecular weight distributions of CHO-rHBsAg and Hans-rHBsAg are shown in Fig. 2. The data clearly show that

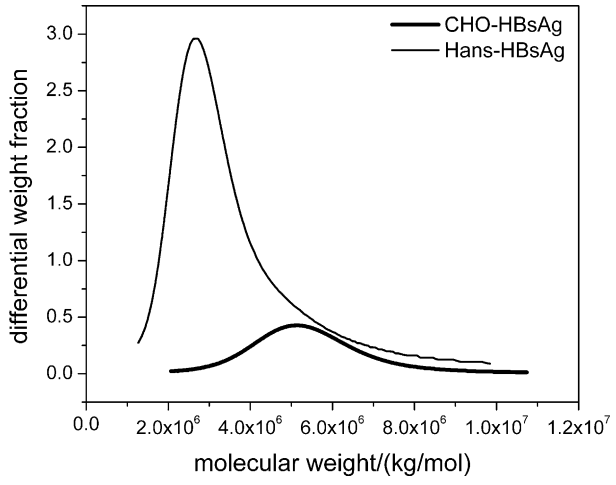

Fig. 2. The differential molecular weight distribution of CHO-rHBsAg (bold line) and Hans-rHBsAg (thin line).

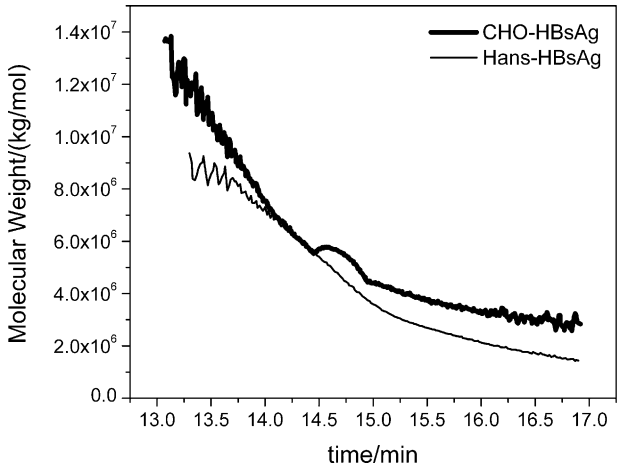

Fig. 3. Plot of molecular weight vs. elution time for CHO-rHBsAg (bond line) and Hans-rHBsAg (thin line).

the CHO-rHBsAg VLP has got larger molecular weight and narrower distribution than the Hans-rHBsAg VLP. Both VLPs are apparently polydisperse. The polydispersity in relation to the particle's homogenicity is characterized by the ratio M_w/M_n [27], where M_n, the number-average molecular weight can be calculated directly using the ASTRA® software. The M_w/M_n values obtained for CHO-rHBsAg and Hans-rHBsAg were 1.07 and 1.15, respectively, demonstrating that CHO-rHBsAg has a slightly more homogeneous structure than Hans-rHBsAg.

Fig. 3 shows the HPSEC plot of the molecular weight versus the elution volume for CHO-rHBsAg and Hans-rHBsAg. The data show that the curves partly coincide in the middle molecular weight region but differ in both the high and low molecular weight regions. This indicates that the structure of the CHO-rHBsAg VLPs may be more compact than that of the Hans-rHBsAg VLPs.

The cumulative molecular weight distribution plots of CHO-rHBsAg and Hans-rHBsAg are shown in Fig. 4. The cumulative molecular weight distribution shows the dependence of the weight fraction as a function of the molecular weight. Even though 71.1% of the CHO-rHBsAg VLPs are distributed

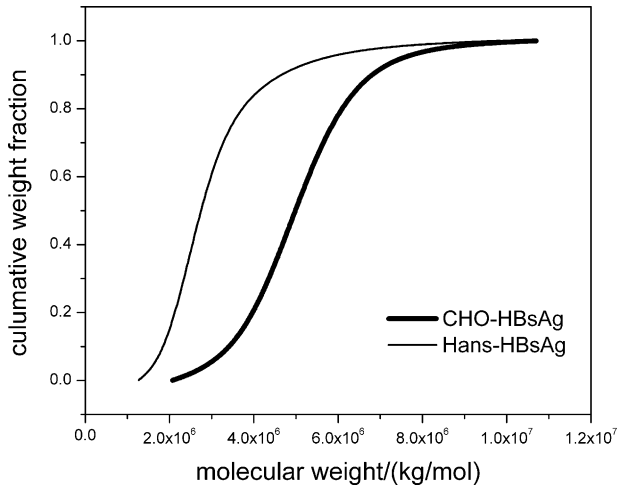

Fig. 4. Plot of cumulative molecular weight distribution for CHO-rHBsAg (bold line) and Hans-rHBsAg (thin line).

between 4000 kDa and 6730 kDa and 71.2% of the Hans-rHBsAg VLPs are distributed between 2050 kDa and 4260 kDa, the CHO-rHBsAg VLPs have got a steeper slope than that of Hans-rHBsAg. This is interpreted as that the distribution of CHO-rHBsAg is narrower than that of Hans-rHBsAg. These data are consistent with the difference in the UV detected elution profile of CHO-rHBsAg and Hans-rHBsAg shown in Fig. 1.

In conclusion, the molecular weight and molecular weight distribution of the CHO-rHBsAg VLPs is larger and more homogeneous than that of Hans-rHBsAg. It is known that the immunogenicity of CHO-rHBsAg is higher than that of Hans-rHBsAg [27]. Besides, the glycosylated structure of CHO-rHBsAg, the effect of a larger molecular weight and a higher homogeneity of the virus-like particles of CHO-rHBsAg may possibly be related to its higher immunogenicity.

3.2. Determination of molecular size and molecular size distributions of recombinant HBsAgs

The retention time of solutes in SEC is not only governed by differences in molecular weights, but also by differences in solute shapes [17]. The relationship between molecular weight and molecular size is not linear and strongly dependent on the solute shape. Information regarding particle shape can be obtained from hydrodynamic measurements of RMS radius (r_g) by light scattering analysis [28]. The RMS of CHO-rHBsAg and Hans-rHBsAg calculated from initial curves of the scattering functions were plotted as a function of the retention time as shown in Fig. 5. The fluctuation of the data at the retention time after about 15 min is the result of the fact that the particle dimensions in this area is probably at the detection limit resulting from a small angular dependence in light scattering. Thus, it has been reported that the lowest detectable radius is about 10 nm for static light scattering [20]. Before the fluctuation part of the retention time line, the RMS radius is descending with each increment of retention time, which corresponds to the data obtained from the molecular weight determination.

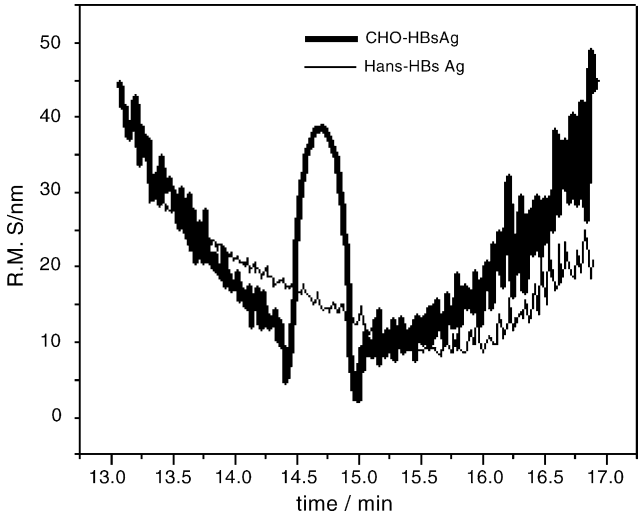

Fig. 5. Plot of RMS radii vs. retention time for CHO-rHBsAg (bold line) and Hans-rHBsAg (thin line).

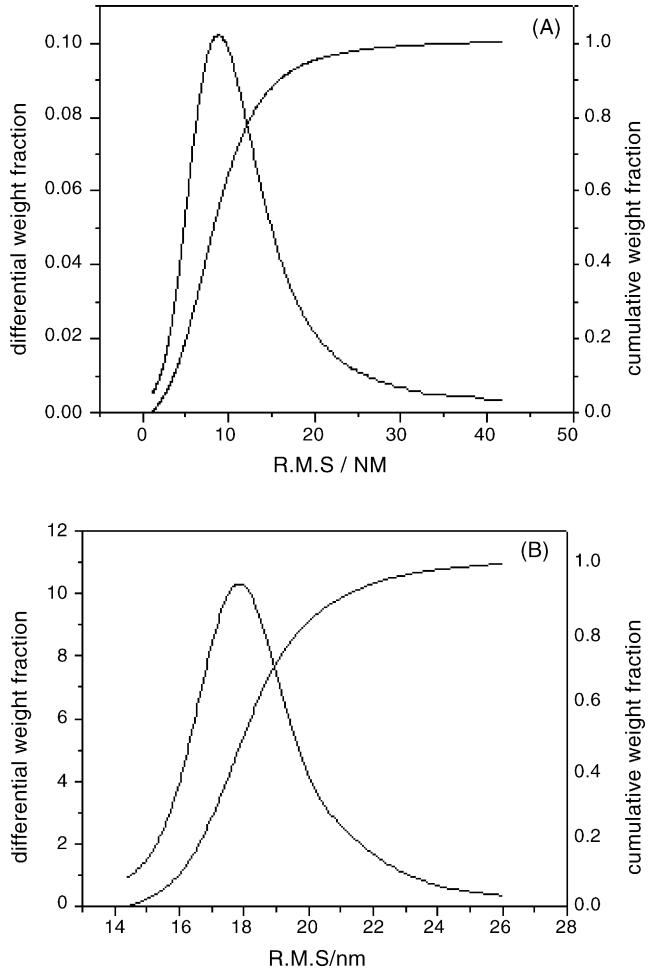

Fig. 6. Plot of differential and cumulative molecular weight distribution for recombinant HBsAg (A) and Hans-rHBsAg (B).

The RMS radius distribution calculated using the ASTRA® software is shown in Fig. 6. The RMS radius of CHO-rHBsAg is distributed from 15 nm to 28 nm, with the average $r_g = 22.1$ nm ($n = 4$), while that of Hans-rHBsAg is distributed from 10 nm to 40 nm, with the average $r_g = 18.1$ nm ($n = 4$). The broader distribution of Hans-rHBsAg compared to that of CHO-rHBsAg correspond with the data obtained by HPSEC, which showed a wider UV peak in the elution curve. The asymmetry of CHO-rHBsAg and Hans-rHBsAg were 0.86 and 0.59, respectively.

The size and size distribution of CHO-rHBsAg are similar to those of plasma-derived HBsAg, with a diameter of 22 nm reported for the Dane particle [29], whereas the Hans-rHBsAg particle shows smaller size and broader size distribution. These data provide further evidence why the CHO-rHBsAg VLPs have better immune response than the Hans-rHBsAg VLPs and thus more effective as a vaccine substance.

3.3. Determination of molecular weights of protein moiety and monomer number of recombinant HBsAgs

The rHBsAg VLPs consist of protein, lipid and carbohydrate. It has been reported that the carbohydrate content is very low and the differences in lipid content of rHBsAg particles do not influ-

Table 2
Calculation M_P of recombinant HBsAg and monomer number of HBsAg

Sample	Batch	(LS)[a]	(UV)[a]	(RI)[a]	ε_P	M_P (kDa)	M of monomer[b]	Monomer number[c]
		3.197[d]	0.588[d]	0.865[d]	0.677[d]	67[d]		1
CHO-rHBsAg	1	12.761	0.172	0.053	3.384	4126	25435	162
	2	13.171	0.176	0.057	3.384	3797	25435	149
	3	12.421	0.168	0.052	3.384	4210	25435	166
	4	13.485	0.179	0.059	3.384	3661	25435	144
Average						3948		155
Hans-rHBsAg	1	6.120	0.114	0.042	3.215	2254	25394	89
	2	9.892	0.173	0.068	3.215	2110	25394	83
	3	9.862	0.178	0.066	3.215	2272	25394	89
	4	8.296	0.153	0.058	3.215	2122	25394	84
Average						2190		86

[a] The integrated area of the absorption peak of the corresponding instrument detector.
[b] The molecular weight of HBsAg monomer calculated from the amino acid composition.
[c] The monomer number calculated from the ratio of M_P to M of monomer.
[d] HSA monomer.

ence their antigenicity and immunogenicity [30]. These essential properties of HBsAg are mainly an effect of the protein moiety, which is characterized by its molecular weight and monomer number of the HBsAg VLP. Since carbohydrate and lipid seldom show absorption at 280 nm, the protein moiety of recombinant HBsAg can be detected by UV, RI and LS detector together and the molecular weight can be calculated using the following equation [21]:

$$M_P = \frac{K_{RI}^2}{K_{LS}K_{UV}} \frac{(LS)(UV)}{\varepsilon_P(RI)^2} \quad (5)$$

where, M_P and ε_P are the molecular weight and extinction coefficient of the protein moiety, respectively. K_{RI}, K_{LS} and K_{UV} are the corresponding instrument calibration constants, and the ratio of $(K_{RI}^2)/(K_{LS}K_{UV})$ can easily be obtained using standard proteins with known molecular weights (M_P) and extinction coefficients (ε_P). The (LS), (UV) and (RI) are the area signals of the corresponding instruments. The (LS) is the area signal of 90° (the 11th angle) of MALLS. The ε_P value can either be obtained from experimental data or estimated with reasonable accuracy from the amino acid composition of the protein monomer.

CHO-rHBsAg and Hans-rHBsAg described here were encoded by the same S sequences and belong to the adr and adw2 sub-type of HBsAg, respectively. The molecular weight of the monomer HBsAg peptide and ε_P calculated from their amino acid sequences were 25435 Da, 3.384 ml/(cm mg) for CHO-rHBsAg and 25394 Da, 3.215 ml/(cm mg) for Hans-rHBsAg [31]. Using human serum albumin monomer with $M_P = 67.0$ kDa and $\varepsilon_P = 0.677$ ml/(cm mg) at 280 nm as standard protein [21], the ratio value of $(K_{RI}^2)/(K_{LS}K_{UV})$ was calculated to be 18071. From the HPSEC chromatograms including signals from the three detectors of LS, RI and UV, the average molecular weight of the protein moiety of CHO-rHBsAg and Hans-rHBsAg, can be obtained using the ASTRA software as shown in Table 2. The results demonstrate that the average molecular weight of the protein moiety of CHO-rHBsAg and Hans-rHBsAg, were 3948 kDa and 2190 kDa ($n = 4$) with standard deviations of 5% and 3%, respectively.

Based on the molecular weights of the protein moiety of the HBsAg VLPs and the monomer HBsAg peptide, calculated from amino acid sequences [31], the monomer number can be calculated. The average monomer numbers obtained was 155 for CHO-rHBsAg and 86 for Hans-rHBsAg particle, respectively. The data coincide with the higher molecular weight of CHO-rHBsAg than that of Hans-rHBsAg. The lower immunogenicity of Hans-rHBsAg may be related to the lower monomer number of Hans-rHBsAg.

4. Conclusion

Currently available recombinant HBV vaccines are immunogenic, efficient and safe. However, the non-response of the vaccinated population of infants and adults is about 5–10%, especially when using the yeast-derived HBsAg vaccine [32]. Knowledge of the particle assembly structure of the Hepatitis B surface antigen from different sources is a prerequisite to understanding the immuno-function mechanism of HBsAg vaccines. New analytical methods that demonstrate the differences between various rHBsAgs are required. To this end, this investigation has successfully explored an accurate, fast and repeatable on-line method using HPSEC-MALLS for comparison of molecular weight, molecular size and their distributions of recombinant Hepatitis B surface antigen derived from CHO and *H. polymorpha* expression systems. This method should be applicable also in the quality control in downstream processing of rHBsAg vaccines, where the assembly structure of rHBsAg VLPs might change by particle aggregation or disassociation on the surface of solid phases.

It is concluded that CHO-rHBsAg VLPs have larger average molecular weights and molecular sizes and narrower molecular size distribution that of Hans-rHBsAg VLPs. In addition, the monomer number is higher for the CHO-rHBsAg VLPs than for the Hans-rHBsAg VLPs. The data provide ample evidence that the higher immunogenicity of CHO-derived HBsAg particles is related to the particles assembly structure and the composition.

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References

[1] W.M. Lee, N. Engl. J. Med. 337 (1997) 1733.
[2] W. Szmuness, C.E. Harley, E.A. Zang, et al., N. Engl. J. Med. 303 (1980) 833.
[3] D. Ganem, H.E. Varmus, Annu. Rev. Biochem. 56 (1987) 651.
[4] J.Y.N. Lau, T.L. Wright, Lancet 342 (1993) 1335.
[5] D. Shouval, J. Hepatol. 39 (2003) S70.
[6] P. Valenzuela, A. Medina, W.J. Rutter, Nature. 298 (1982) 347.
[7] E. Hardy, D. Martinez, D. Diago, R. Diaz, D. Gonzalez, L. Herrerz, J. Biotech. 77 (2000) 157.
[8] G. Gellissen, Z.A. Janowicz, U. Weydemamm, et al., Biotech. Adv. 10 (1992) 179.
[9] M.L. Michel, P. Pontisso, E. Sobczak, et al., Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 7708.
[10] D. Shouval, Y. Ilan, R. Adler, et al., Vaccine 12 (1994) 1453.
[11] D. Diminsky, R. Schirmbeck, J. Reimann, Y. Barenholz, Vaccine 15 (1997) 637.
[12] D. Tleugabulova, V. Falcón, E. Pentón, J. Chromatogr. A 845 (1999) 171.
[13] F. Gavilanes, J.M. González-Ros, D. Lee, J. Biol. Chem. 257 (1982) 7770.
[14] F. Gavilanes, J. Gómez-Gutierrez, J. Aracil, et al., Biochem. J. 265 (1990) 857.
[15] G.A. Cabral, J. Gen. Virol. 38 (1978) 339.
[16] Y. Sato, N. Ishikawa, T. Takagi, J. Chromatogr. 507 (1990) 25.
[17] M.J. Potschake, J. Chromatogr. 441 (1998) 239.
[18] T. Takagi, J. Chromatogr. 592 (1990) 409.
[19] P.J. Wyatt, Anal. Chim. Acta 272 (1993) 1.
[20] P.J. Wyatt, J Chromatogr. 648 (1993) 27.
[21] J. Wen, T. Arakawa, J.S. Philo, Anal. Biochem. 240 (1996) 155.
[22] A. Olivia, M. Llabrés, J.B. Fariña, J. Pharma. Biomed. Anal. 25 (2001) 833.
[23] J. Barackman, I. Prado, C. Karunatilake, K. Furuya, J. Chromatogr. A. 1043 (2004) 57.
[24] M. Belew, Y.F. Mei, B. Li, J. Berglöf, J.-C. Janson, Bioseparation 1 (1991) 397.
[25] Z.Y. Hu, D.G. Wang, Z.L. Zheng, et al., Prog. Microb. Immun. 30 (2002) 36.
[26] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[27] D. Tleugabulova, J. Reyes, L. Costa, et al., Chromatographia 45 (1997) 317.
[28] J.C. Janson, L. Rydén, Protein Purification-Principles, High-Resolution Methods, and Applications, Wiley-LISS, New York, 1998.
[29] P. Maupas, A. Goudeau, P. Coursaget, J. Drucker, Lancet 1 (1976) 1367.
[30] J. Pêtre, F.V. Wijnendaele, B.D. Neys, et al., Postgrad. Med. J. 63 (Suppl. 2) (1987) 73.
[31] www.expasy.org.
[32] C.A. Alper, Exp. Clin. Immunogenet. 12 (1995) 171.