The Frontiers of Society, Science and Technology
ISSN 2616-7433 Vol. 2, Issue 13: 27-38, DOI: 10.25236/FSST.2020.021304

Effect of Aluminum Adjuvant on the Structure of Recombinant Hepatitis B Core Antigen

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Abstract. *Adjuvants are important for vaccines to reduce the amount of antigen used and improve the immune response ability of antigens. However existence of adjuvants may sometimes alter the antigen structures and subsequently affect their immunogenicity. The effect of aluminum hydroxide adjuvant on the structure of recombinant hepatitis B core antigen (HBcAg) was studied. Differential scanning fluorimetry (DSF) showed that the melting temperature (T_m) of HBcAg was significantly decreased for 5.4 ℃ by adsorbing to aluminum hydroxide adjuvant, indicating adjuvant will reduce the thermal stability of HBcAg. The adsorbed HBcAg on aluminum hydroxide adjuvant was released with 1 M NaCl and was analyzed by high performance size exclusion chromatography (HPSEC). HPSEC showed the antigens partially aggregated and dissociated. Transmission electron microscopy (TEM) further showed that the adsorbed HBcAg became ellipse, suggesting some distortion occurred. Furthermore, the effect of storage temperature for adjuvanted HBcAg was evaluated at -20 °C, 4 °C, and 37 °C. The T_m was all decreased as compared to free antigen. The T_m of 89.0 °C was the highest at 4 °C, indicating the HBcAg was the most stable under this storage condition. The study indicated adsorption of HBcAg to aluminum hydroxide adjuvant will cause some structural changes and reduce its thermal stability. Therefore the influence of adjuvant on antigen cannot be ignored in formulation research.*

Keywords: *HBcAg, Aluminum adjuvant, Stability, Structure, Differential scanning fluorimetry*

1. Introduction

Adjuvant can nonspecifically enhance or change the immune response of the body to an antigen. Early vaccines consisted of inactivated whole bacteria, attenuated or inactivated viruses or bacterial toxoids. These vaccines are not as pure as those prepared using modern protein purification techniques. However, these impure vaccines often contain "intrinsic adjuvants", which include traces of active exotoxins or endotoxins. With the increasing demand for the safety of vaccines, vaccines are demanded towards higher purity and clearer composition. However, the purification of vaccine antigen often leads to the elimination of the adjuvant effect of

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vaccine, which can reduce the immunogenicity of highly purified vaccine antigen.

The use of immune adjuvants is the most promising strategy for enhancing the protective immune response of these vaccines. Immune adjuvants enhance the uptake, processing and presentation of antigens by antigen presenting cells, especially dendritic cells, which are the only presenting cells capable of presenting antigens to initial T cells [1]. Adjuvants that induce dendritic cell maturation enhance the immune response by activating T cells. Aluminum salt adjuvants are the most widely used adjuvants in human vaccines [2]. Aluminum adjuvants that have been used include aluminum hydroxide adjuvant, aluminum phosphate adjuvant and alum. The best known aluminum hydroxide adjuvant is Alhydrogel® and aluminum phosphate adjuvants are AdjuPhos®.

The major problem of using adjuvant in vaccine production is that it may affect the immune efficacy of the vaccine [3]. The structure of an antigen is closely related to its biological activity, and changes in structure may lead to changes in biological activity. Nevertheless, there are interfacial adsorption and electrostatic interactions between adjuvants and antigens, which may have a significant impact on the structure of antigens [4]. It was reported both model protein antigens like lysozyme and those with more complex structure such as virus and virus like particles (VLPs) may be structurally altered, or become less stable, after adsorption onto aluminum salt adjuvants [4-6]. The presence of multiple interfaces and surfactants in emulsion adjuvants is also a challenging environment for antigens [4]. The inactivated foot-and-mouth disease virus (iFMDV), which is also known as 146S, was reported gradually dissociate into smaller 12S particles within 2-3 months of storage at 4°C in oil-emulsion adjuvant [7], resulting in significant loss of immune activity. Therefore, the effect of adjuvants on the structure of antigens needs deeper study.

In this study, the effect of aluminum hydroxide adjuvant on the structure of recombinant hepatitis B core antigen (HBcAg) was studied. HBcAg is the most fascinating virus like particle as a vaccine platform, due to its high immunogenicity and extraordinary flexibility allowing insertion of up to 300 amino acids without affecting its ability to self-assemble into VLPs structure [8]. The thermal stability of HBcAg was analyzed by differential scanning fluorimetry (DSF), the particle integrity of HBcAg on and released from adjuvant was analyzed by transmission electron microscopy (TEM) and high performance size exclusion chromatography (HPSEC), respectively. Our results showed that aluminum hydroxide adjuvant will reduce the stability of HBcAg, therefore the effect of adjuvants for antigens should not be ignored. The study provides guidance for vaccine development.

2. Materials and Methods

2.1 Materials

Aluminum hydroxide adjuvant was purchased from Sigma-Aldrich (MO, USA). Pure HBcAg, with purity greater than 95%, were prepared and kindly provided by Institute of Process Engineering, Chinese Academy of Sciences (China, Beijing). To

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prepare the antigen, firstly, *E. coli* fermentation bacteria were collected and sonicated. After centrifugation, the cell fragments were removed and the supernatant containing antigen was harvested. The purification was performed as reported previously by chromatography [9]. BCA kits were purchased from Thermo Company, and Sypro Orange fluorescent dye was purchased from Sigma-Aldrich (MO, USA). Other reagents were analytical reagents.

2.2 Study on adsorption of HBcAg by aluminum hydroxide adjuvant

In order to ensure that there will not be excessive antigen not being adsorbed by the adjuvant and lead to the deviation of experimental results, we need to ensure that the proportion of adjuvant and antigen is matched. The adsorption capacity of HBcAg on aluminum hydroxide adjuvant was measured. Under the condition of 20 mM pH 7.4 sodium phosphate buffer with or without 0.15 M NaCl, 1 mL of 0.25 mg/mL HBcAg was mixed with different amounts of aluminum adjuvant from 0 to 1 mL. The rest of the volume was supplemented with buffer to the total volume of 2 mL for each sample. After incubation for 60 min at room temperature on a rotator, the mixtures were centrifuged at 8000 rpm for 5 min. BCA method was used to detect the protein concentration in the supernatant.

2.3 Determination of protein concentration using BCA method

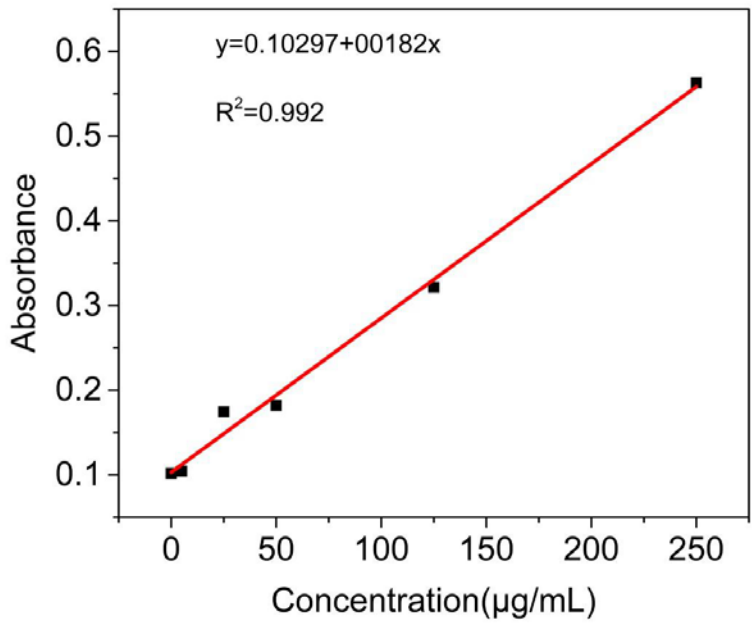

Figure 1. The calibration curve of protein concentration versus OD 562

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The protein concentration was determined by BCA method. Briefly, the reagent A and reagent B was mixed at a ratio of 50:1. Then 200 μL of the mixtures was added in a 96 well plate for each tube, and 20 μL of sample was added to each well and incubated at 37℃ for 30 min. The absorbance at 562 nm was used to calculate protein concentration. A calibration curve of protein concentration versus OD 562 was built by a series of BSA standards from 0 to 250 μL/mL, with a good linear response (R^2=0.992, Fig. 1). Each assay was triplicated.

2.4 Differential scanning fluorimetry analysis

DSF was used to determine the change of melting temperature, T_m, before and after HBcAg adsorbing to aluminum hydroxide adjuvant. DSF analyses were performed with an ABI 7500 Fast RT-PCR instrument (Applied Biosystems, USA) as reported. The instrument uses a tungsten halogen lamp as light resource with five fixed filters.

For detection, 40 μL of free HBcAg or adsorbed HBcAg was added with 2 μL of Sypro Orange fluorescent dye diluted by 10 times. The mixtures were then loaded to 96-well polypropylene plates (Applied Biosystems, USA) and capped with optical cap strips (Sorenson BioScience Inc., USA) to prevent sample evaporation. The plates were heated from 25 to 95°C at a scan rate of 1 °C/min, and three repeated measurements were performed for each sample. The fluorescence was collected using ROX filter. Melting temperature (T_m) was calculated according to the maximum of the first derivative plot of the fluorescence curve.

2.5 Particle integrity of HBcAg analyzed by HPSEC

To verify whether the HBcAg was kept intact after adsorption to aluminum hydroxide adjuvant, the adsorbed HBcAg was released with 1 M NaCl and was then analyzed by HPSEC. The chromatograms of HBcAg before and after adsorption were compared. The HPSEC analysis was performed on an Agilent 1260 HPLC system equipped with TSK G5000 PW_{XL} column (300×7.8 mm, I.D.). The mobile phase was 50mM sodium phosphate (pH 7.0). The analysis was carried out at a flow rate of 0.5 mL/min, and the effluent was monitored with a variable wavelength detector at 280 nm. In order to ensure that NaCl will not affect the structure of HBcAg, free HBcAg added with 1 M NaCl was also analyzed as a control.

2.6 Transmission electron microscopy

The structures of free HBcAg and HBcAg adsorbed on aluminum hydroxide adjuvant were observed by Philips FEI Tecnai 20 transmission electron microscopy (TEM, Royal Philips Electronics, Amsterdam). The samples (0.25 mg/mL) were applied to a 400-mesh copper grid, after removing excess solutions by using filter paper. The specimen was negatively stained with 1% (v/v) phosphotungstic acid for 1 min.

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2.7 Effect of storage temperature on adjuvanted HBcAg

To study the effect of storage temperature on adjuvanted HBcAg, the adsorbed antigens were placed at -20 °C, 4 °C, and 37 °C for 24 h, respectively. The thermal stability was then analyzed by DSF to detect the change of their structures.

3. Results and Discussions

3.1 Adsorption of HBcAg on aluminum hydroxide adjuvant

From the study on the adsorption of HBcAg by aluminum hydroxide adjuvant, the protein adsorption capacity (AC) and adsorption efficiency (AE) under different adjuvant concentrations were firstly calculated and shown in Fig. 2. The adsorption efficiency was calculated as follows,

$$\text{AE}\ (\%) = (m_t - m_s) / m_t \times 100 \quad (1)$$

Where m_t is the total amount of antigen added into the system, and m_s is the amount of antigen in the supernatant. The adsorption capacity was calculated as follows:

$$\text{AC}\ (\text{mg/mL}) = (m_t - m_s) / V_a \quad (2)$$

Where m_t is the total amount of antigen added to the system, m_s is the amount of antigen in the supernatant, V_a is the amount of aluminum hydroxide adjuvant.

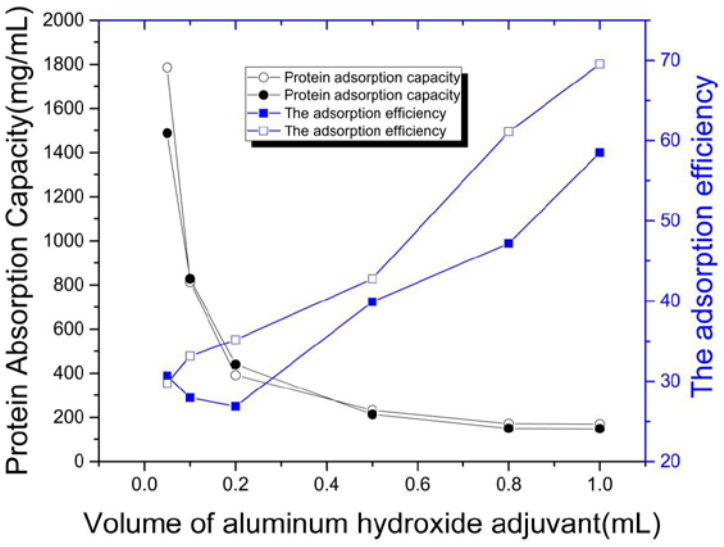

Figure 2. The relationship between protein adsorption capacity/efficiency and the volume of aluminum hydroxide adjuvant

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As increasing the volume of aluminum hydroxide adjuvant, the AE increased gradually while AC decreased gradually. In other words, the AE of antigen was in direct proportion to the dosage of aluminum hydroxide adjuvant, while the amount of protein adsorption was inversely proportional to the adjuvant added. The results were consistent under conditions with or without 0.15 M NaCl. The highest AE of about 70% was obtained at appending 1 mL adjuvant with 1 mL antigen. The following studies were therefore conducted under this condition.

3.2 Effect of aluminum hydroxide adjuvant on the thermal stability of HBcAg

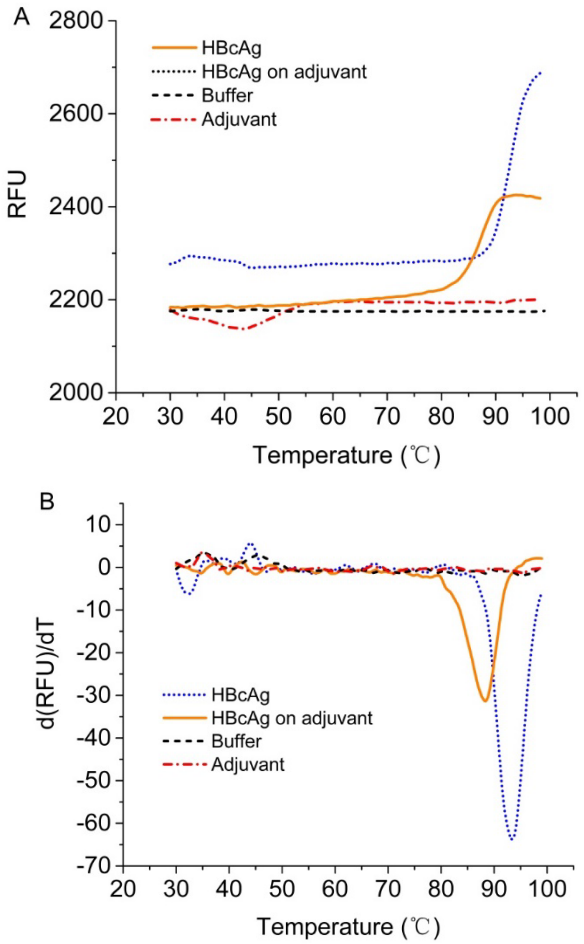

Figure 3. DSF analyses of the Tm values of free HBcAg and HBcAg on adjuvant. (A) Recording of fluorescence intensities as a function of temperature. (B) The plot of first derivative of the fluorescence intensities d(RFU)/dT as function of temperature.

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The thermal stability of HBcAg on adjuvant was analyzed in an on-line mode by DSF. DSF is applied to determine the melting temperature of proteins by using small molecule fluorescent compounds combining to proteins by covalent or non-covalent interactions to form strong fluorescence. The Sypro Orange used in this study combines the exposed hydrophobic regions during the denaturation of proteins. The advantage of DSF is that the existence of adjuvant will affect signals of most analytical techniques, while DSF will not be influenced due to the detection is usually conducted at 90◦ or from the top of the multi-well plates [10]. Therefore DSF is a suitable technique to study the structural changes of antigens on adjuvants.

The buffer and aluminum hydroxide adjuvant were analyzed as controls. From the signals in Fig. 3A, they showed no signal changes, indicating buffer and adjutant have no influence on DSF detection. For HBcAg samples, a sigmoidal increase in the fluorescence signal and subsequent decrease after thermal transition was observed for both free HBcAg and HBcAg on adjuvant. The increase of signals indicated the unfolding of HBcAg during thermal scanning [11]. To determine the thermal transitions temperature, T_m, first-order derivative of the fluorescence signal was plotted as presented in Fig. 3B.

The T_m of free HBcAg was determined to be 93.6 °C. Such high T_m indicated HBcAg is a highly stable particle, which is probably due to the abundant disulfide bonds in HBcAg [12]. The high stability also reflects its advantages as an antigen or antigen delivery system. Nevertheless, HBcAg with adjuvant had a T_m of 88.2 °C, showing a significant decrease of 5.4 °C. This result indicated adsorption to aluminum hydroxide adjuvant did alter the structure of HBcAg that it significantly reduced the thermal stability of HBcAg.

3.3 Particle integrity of HBcAg on aluminum hydroxide adjuvant

To further study if the aluminum hydroxide adjuvant will affect the particle integrity of HBcAg, the adsorbed antigens were released with 1 M NaCl and were analyzed by HPSEC. Since HPSEC separates molecules by size that the larger components will be eluted quicker than the small components, aggregation or dissociation of the HBcAg can be detected by HPSEC according to the change of elution time. The chromatograms are shown in Fig. 4.

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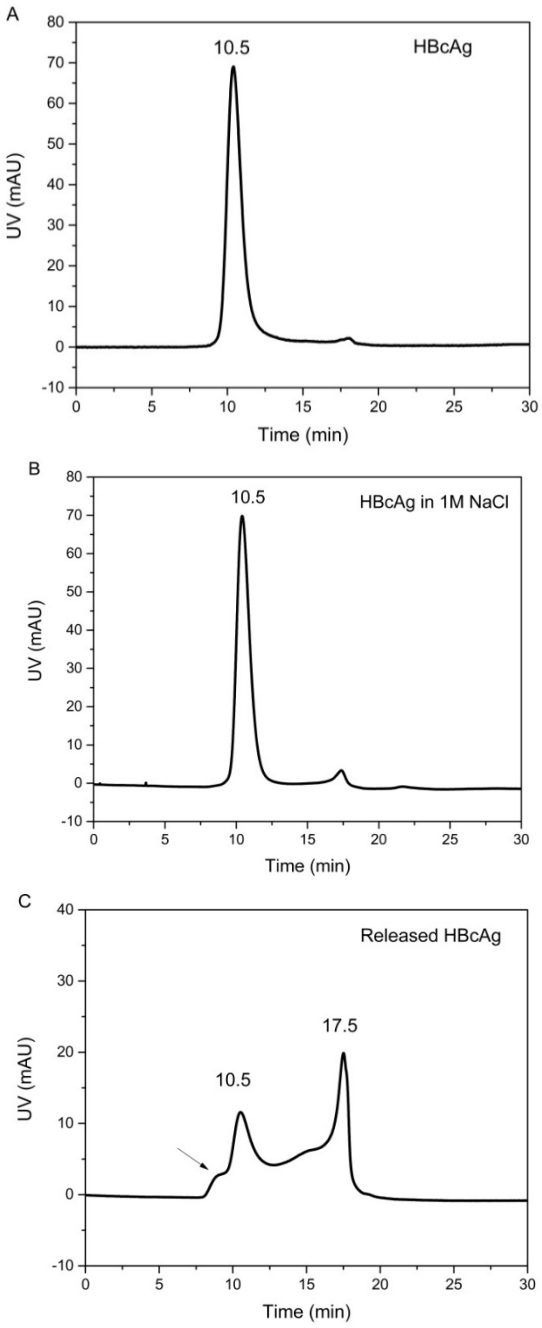

Figure 4. HPSEC chromatograms of (A) free HBcAg, (B) HBcAg in 1M NaCl, and (C) HBcAg released from aluminum hydroxide adjuvant with 1M NaCl.

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There is only one absorption peak of HBcAg at 10.5 min in HPSEC (Fig. 4A), indicating the purity of HBcAg and the peak position of intact particles. The chromatogram of HBcAg released by 1 M NaCl is shown in Fig. 4C. In addition to the absorption peak of HBcAg at 10.5 min, a small shoulder peak appeared before 10.5 min, and a large absorption peak appeared at about 17.5 min. In order to verify whether the changes of HBcAg were caused by 1 M NaCl, the results of adding 1 M NaCl into pure HBcAg are shown in Fig. 4B. The peak was still about 10.5 min, indicating that the changes of released HBcAg was not caused by 1 M NaCl but by adsorption to aluminum hydroxide adjuvant. According to the detection principle of HPSEC, the results showed that the released HBcAg was partially aggregated and depolymerized after releasing from aluminum adjuvant. This implied that the adsorption to aluminum hydroxide adjuvant may lead to the destruction of particle structure of HBcAg. The yield of antigen was. By comparing the peak area at 10.5 min, the recovery of released intact HBcAg was only 17.6%.

TEM was conducted to further detect the particle integrity on adjuvant. As shown in Fig. 5A, the free HBcAg exhibited a circular shape of about 27 nm. However, the HBcAg adsorbed on aluminum hydroxide adjuvant (Fig. 5B) showed some distortion of the structure that it became ellipse.

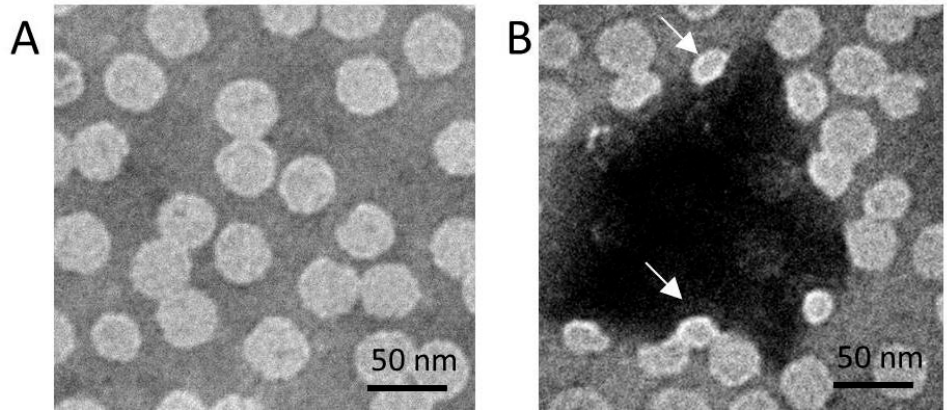

Figure 5. TEM of (A) HBcAg and (B) HBcAg adsorbed on aluminum hydroxide adjuvant

3.4 Effect of storage temperature on stability of adjuvanted HBcAg

The adjuvanted HBcAg was stored at -20 °C, 4 °C, and 37 °C for 24 h, respectively, to evaluate the effect of temperature on the antigens.

The -20 °C group obviously precipitated compared with the other two groups after thawing. It has been reported that freeze-thawing will make adjuvant particles denatured [13]. After that, the adjuvanted HBcAg was analyzed by DSF. Typical signals of the samples at three different temperatures are presented in Fig. 6. As we

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can see in Fig. 6, the T_m of -20 °C group was significantly lower than that of the other two groups, which was only 82.6 °C. The T_m of 4 °C and 37 °C groups was 89.0 °C and 87.4 °C, respectively. Compare with free HBcAg which has a T_m of 93.6 °C as determined previously, all three adjuvanted HBcAg had decreased stability, suggesting structural changes occurred. However, antigens stored at 4 °C had the highest T_m, indicating the adjuvanted antigens were the most stable at this storage temperature. Therefore choosing the right storage temperature is also essential to keep the adjuvanted antigens stable.

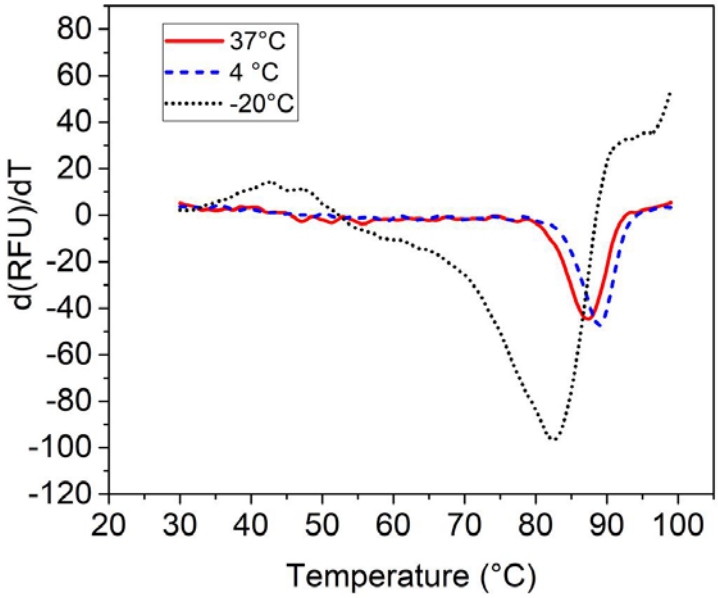

Figure 6. Melting temperature of adjuvanted HBcAg after stored at different temperatures detected by DSF

4. Conclusions

In this work, the effect of aluminum hydroxide adjuvant on the structure of recombinant hepatitis B core antigen was studied. Through on-line DSF analyses, it was found that adsorption to aluminum adjuvant will decrease the thermal stability of HBcAg antigen, indicating some structural changes occurred on the adjuvant. Through HPSEC and TEM, it was found that aluminum adjuvant could also affect the intact particle structures of HBcAg.

The effect of adjuvants on the antigenic structure has been found in some studies. In this paper, we also show that adjuvants may destroy the antigenic structure. Despite the use of adjuvants will release the antigen slowly, prolong the time of antigen action, and strengthen the immune capacity of the immune system, they may

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also have some adverse effects on antigen structures and even *in vivo* immunogenicity. Our results indicate that the influence of adjuvant on antigen cannot be ignored in formulation research. Therefore, we need to do more research on the stability of vaccine to ensure more stable antigen, such as improve the stability of vaccine antigens through changing the solution conditions and to select adjuvant that is beneficial to the stability of the antigens.

Acknowledgements

The author would like to thank Dr. Yang from the Chinese Academy of Sciences for her guidance and help.

References

[1] F. Azmi, A.A.A. Fuaad, M. Skwarczynski, I. Toth, Recent progress in adjuvant discovery for peptide-based subunit vaccines, Hum Vacc Immunother, 10 (2014) 778-796.

[2] L.A. Brito, P. Malyala, D.T. O'Hagan, Vaccine adjuvant formulations: A pharmaceutical perspective, Semin Immunol, 25 (2013) 130-145.

[3] J.P. Nkolola, A. Cheung, J.R. Perry, D. Carter, S. Reed, H. Schuitemaker, M.G. Pau, M.S. Seaman, B. Chen, D.H. Barouch, Comparison of multiple adjuvants on the stability and immunogenicity of a clade C HIV-1 gp140 trimer, Vaccine, 32 (2014) 2109-2116.

[4] B. Hansen, M. Belfast, G. Soung, L. Song, P.M. Egan, R. Capen, H. Hogenesch, R. Mancinelli, S.L. Hem, Effect of the strength of adsorption of hepatitis B surface antigen to aluminum hydroxide adjuvant on the immune response, Vaccine, 27 (2009) 888-892.

[5] L.S. Jones, L.J. Peek, J. Power, A. Markham, B. Yazzie, C.R. Middaugh, Effects of Adsorption to Aluminum Salt Adjuvants on the Structure and Stability of Model Protein Antigens, J Biol Chem, 280 (2005) 13406-13414.

[6] M. Regnier, B. Metz, W. Tilstra, C. Hendriksen, W. Jiskoot, W. Norde, G. Kersten, Structural perturbation of diphtheria toxoid upon adsorption to aluminium hydroxide adjuvant, Vaccine, 30 (2012) 6783-6788.

[7] M.M. Harmsen, H.P.D. Fijten, D.F. Westra, A. Dekker, Stabilizing effects of excipients on dissociation of intact (146S) foot-and-mouth disease virions into 12S particles during storage as oil-emulsion vaccine, Vaccine, 33 (2015) 2477-2484.

[8] A.Z. Baltabekova, Z.S. Shagyrova, A.S. Kamzina, M. Voykov, Y. Zhiyenbay, E.M. Ramanculov, A.V. Shustov, SplitCore Technology Allows Efficient Production of Virus-Like Particles Presenting a Receptor-Contacting Epitope of Human IgE, Mol Biotechnol, 57 (2015) 746-755.

[9] Z.J. Li, J.X. Wei, Y.L. Yang, X.W. Ma, B.D. Hou, W.Q. An, Z.L. Hua, J.J. Zhan, Y. Li, G.H. Ma, S.P. Zhang, Z.G. Su, Strong hydrophobicity enables efficient purification of HBc VLPs displaying various antigen epitopes through hydrophobic interaction chromatography, Biochem Eng J, 140 (2018) 157-167.

ISSN 2616-7433 Vol. 2, Issue 13: 27-38, DOI: 10.25236/FSST.2020.021304

[10] Y. Yang, Z. Su, G. Ma, S. Zhang, Characterization and stabilization in process development and product formulation for super large proteinaceous particles, Eng Life Sci, n/a (2020).
[11] B.E. Lang, K.D. Cole, Differential Scanning Calorimetry and Fluorimetry Measurements of Monoclonal Antibodies and Reference Proteins: Effect of Scanning Rate and Dye Selection, Biotechnol Progr, 33 (2017) 677-686.
[12] J. Zheng, F. Schodel, D.L. Peterson, The Structure of Hepadnaviral Core Antigens - Identification of Free Thiols and Determination of the Disulfide Bonding Pattern, J Biol Chem, 267 (1992) 9422-9429.
[13] V.A. Solanki, N.K. Jain, I. Roy, Stabilization of tetanus toxoid formulation containing aluminium hydroxide adjuvant against freeze-thawing, Int J Pharmaceut, 414 (2011) 140-147.