Hepatitis B core (HBc) virus like particle (VLP) as a platform for innovation of chimeric adjuvant-free VLP vaccines targeting oncoviruses

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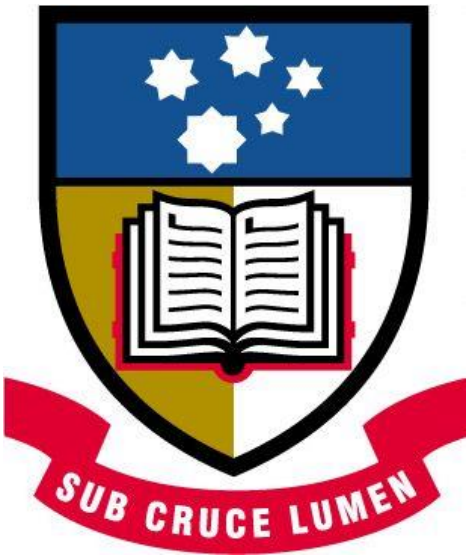
A thesis submitted for the degree of Doctor of Philosophy



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Abbreviations

B/IC: baculovirus/insect cell

CD: circular dichroism

CHO cell: Chinese hamster ovary cell

cryoEM: cryo electron microscopy

CVA6: coxsackievirus A6

DSC: differential scanning calorimeter

E. coli: *Escherichia coli*

EBNA1: Epstein-Barr nuclear antigen 1

EBV: Epstein-Barr virus

GPI: glycosylphosphatidylinositol

HAV: hepatitis A virus

HBc: hepatitis B core

HBsAg: hepatitis B surface antigen

HCV core: hepatitis C virus core

HCV: hepatitis C virus

HEK293 cell: human Embryonic Kidney 293 cell

HEV: hepatitis E virus

HEV: hepatitis E virus

HPSEC-MALLS: high-performance size-exclusion chromatography-multi-angle static laser light scattering

HPV: Human papillomavirus

HTLV-1: T-cell leukaemia virus type 1

IF: intrinsic fluorescence

IHHNV: necrosis virus

KSHV: Kaposi's sarcoma-associated herpesvirus

MC: Monte Carlo

MD: molecular dynamic

MDSCs: myeloid derived suppressor cells

MuPyV: murine polyomavirus VP1

NDV: Newcastle disease virus

NMR: nuclear magnetic resonance

OPV: oral polio vaccine

PAMP: pathogen associated molecular pattern

PCV2: porcine circovirus type 2

PEI: polyethyleneimine

PTM: post-translational modification

PVY: potato virus Y

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SUMO: small ubiquitin-like modifier

TAM: tumour-associated macrophage

TEM: transmission electron microscopy

TME: tumour microenvironment

Tregs: regulatory T cells

VLP: virus like particle

Abstract

Immunotherapy is an advanced technology for treatment of oncoviruses leading cancers. However, lack of effective and safe vaccines against the oncoviruses has limited the development. This thesis aims to apply Hepatitis B core (HBc) virus like particle (VLP) as a platform for innovation of chimeric adjuvant-free VLP vaccines targeting oncoviruses. Two chimeric HBc VLP-based vaccines presenting Epstein–Barr virus nuclear antigen 1 (EBNA1) epitope (short and non-structural epitope) and Hepatitis C virus (HCV) core epitope (long and structural epitope) were successfully expressed and purified in the *Escherichia coli* (*E. coli*) expression system with high production yields, 62.1 mg/g and 40.3 mg/g of wet cell weight, respectively.

To further understand and evaluate the influence of insertion of different epitopes to HBc VLP, the stability of chimeric HBc VLP vaccines under different stresses were analysed in comparison with non-chimeric HBc VLP. Computational protein modelling was employed to assist the understanding of the possible cause for the differences. Results indicate that the stability of chimeric HBc VLP vaccines was related to the hydrophobicity of chimeric HBc monomers. The stability of chimeric HBc VLP decreased with the decrease of hydrophobicity of its monomer. This finding would help and improve the efficiency in the development and design of chimeric HBc VLP-based vaccines.

In the immunogenicity evaluation, both adjuvant-free EBNA1-HBc VLP and HCV core-HBc VLP induced strong epitope-specific immune response in mice compared

with other reported vaccine candidates of EBV and HCV. The achieved immune responses of adjuvant-free EBNA1-HBc VLP and HCV core-HBc VLP groups were comparable to the groups with aluminium adjuvant. No side effect and death of mice were detected during the examination. This confirms that adjuvant-free HBc VLP can present either short non-structured epitope or long structured epitope and can induce strong epitope-specific immune response with low safety risks. Chimeric EBNA1-HBc VLP tended to elicit predominated humoral immune response, while chimeric HCV core-HBc VLP induced predominated cellular immune response. This indicates that the nature of antigens presented by HBc VLP has an impact on the immune response performance, which should be considered in the design of chimeric HBc VLP vaccines in the future. The thesis also found that the addition of aluminium adjuvant would improve the humoral immune response while supressing the cellular immune response of chimeric HBc VLP vaccines. EBNA1-HBc VLP was less affected by the adjuvant on the immune response tendency compared with HCV core-HBc VLP. At last, long-term immunogenicity of two chimeric HBc VLPs were examined by evaluated the epitope specific memory T cells. Both HCV core-HBc VLP and EBAN1-HBc VLP showed good potential for long-term protection.

With all above findings, chimeric adjuvant-free HBc VLP-based vaccine is promising to present different types of oncoviruses epitopes with high epitope-specific immune response and low risks. More epitopes targeting different oncoviruses could be presented by chimeric adjuvant-free HBc VLP platform for cancer treatment, and further computational protein modelling is helpful in the design and investigation of

these novel chimeric HBc VLP-based vaccines.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Chapter 1 Introduction

1.1 Background

Cancers have caused the death of millions of people globally and remain a severe issue in modern society [8]. Cancer, also known as “Pathology of the Century”, has also been defined as the “the modern disease par excellence” or even the “the quintessential product of modernity” [9-11]. From the eighteenth century to now, cancers have become the second major cause of death worldwide [12]. In this case, different treatments against cancers have been developed. Traditional approaches include surgery, chemotherapy and radiation therapy [13]. However, these traditional treatment methods have several drawbacks such as pain and infection to the patient, damages to patients’ body and side effects.

Recently, researchers have proven that a kind of viruses, oncovirus, is related to around 16 % of 38 different types of cancers [14]. These oncoviruses are studied as targets for the treatment and prevention of cancers in the development of immunotherapy. Immunotherapy, is a kind of novel approach to treat cancers by using patients’ own immune system for cancer prevention or to fight against cancer cells [15]. Researchers have employed different platforms to develop cancer vaccines, such as virus-like particles (VLPs) [16], ferritin [17] and nanoparticles [18] to prevent and treat the infections which potentially lead to cancers [19].

Among these platforms, Hepatitis B core virus-like particle (HBc VLP) platform is one of the most popular candidates and has been adopted for the application for carrying

foreign epitopes in the last decade [20-22]. HBc VLP platform has drawn the interest of researchers due to its unique structure and merits [23, 24]. HBc VLP has a repetitive antigenic structure that could present the foreign epitope in a high density and trigger strong epitope specific immune response due to its self-adjuvant property [16, 25-27]. In addition, HBc VLP has no viral genome, which makes it a safer vaccine candidate compared with the traditional viral vaccines [28, 29]. Moreover, HBc VLP-based vaccines can be produced in multiple expression systems [30, 31].

To produce effective VLP-based vaccines presenting foreign epitopes from oncoviruses, the development of recent technology and challenges of VLP-based vaccines were reviewed. Several challenges have been identified including 1) to design and present different types of foreign antigens on the surface of chimeric VLP, 2) to perform effective soluble expression and purification of designed chimeric VLP proteins to achieve high production yield using suitable expression system, 3) to maintain assembled VLP structure of produced chimeric VLP proteins with good stability to ensure its immunogenicity and 4) to achieve strong and long-term antigen specific immunogenicity of produced chimeric VLP vaccine *in vivo*.

1.2 Aim and Objectives

The aim of this project is to develop effective adjuvant free chimeric HBc VLP derived vaccines by applying HBc VLP platform to present foreign epitopes targeting oncoviruses. To achieve this aim, this project focuses on objectives as follows:

1. To optimize the soluble expression and purification of designed chimeric HBc

VLPs presenting different foreign epitopes targeting Hepatitis C virus (HCV) and Epstein–Barr virus (EBV) using *E. coli* expression system in high production yields,

2. To study and understand the possible mechanism that can influence on the stability and the assembly of produced chimeric HBc VLPs,

3. To evaluate the *in vivo* immunogenicity of two produced chimeric HBc VLP to confirm their performance of effective and long-term antigen specific immune response.

The process of this project is illustrated in **Figure 1.1**. EBV and HCV epitope, Epstein-Barr nuclear antigens 1 (EBNA1), and Hepatitis C virus core (HCV core), were fused to HBc VLP protein to form chimeric EBNA1-HBc and HCV core-HBc VLPs. The designed recombinant chimeric HBc VLPs were expressed and purified using *E.coli* expression system. The characterizations and properties of expressed chimeric HBc VLPs were investigated systematically. Then the immunogenicity performance and stability of produced chimeric HBc VLPs were evaluated, and computational protein modelling and molecular dynamic (MD) simulation were employed for the evaluation of the stability tests.

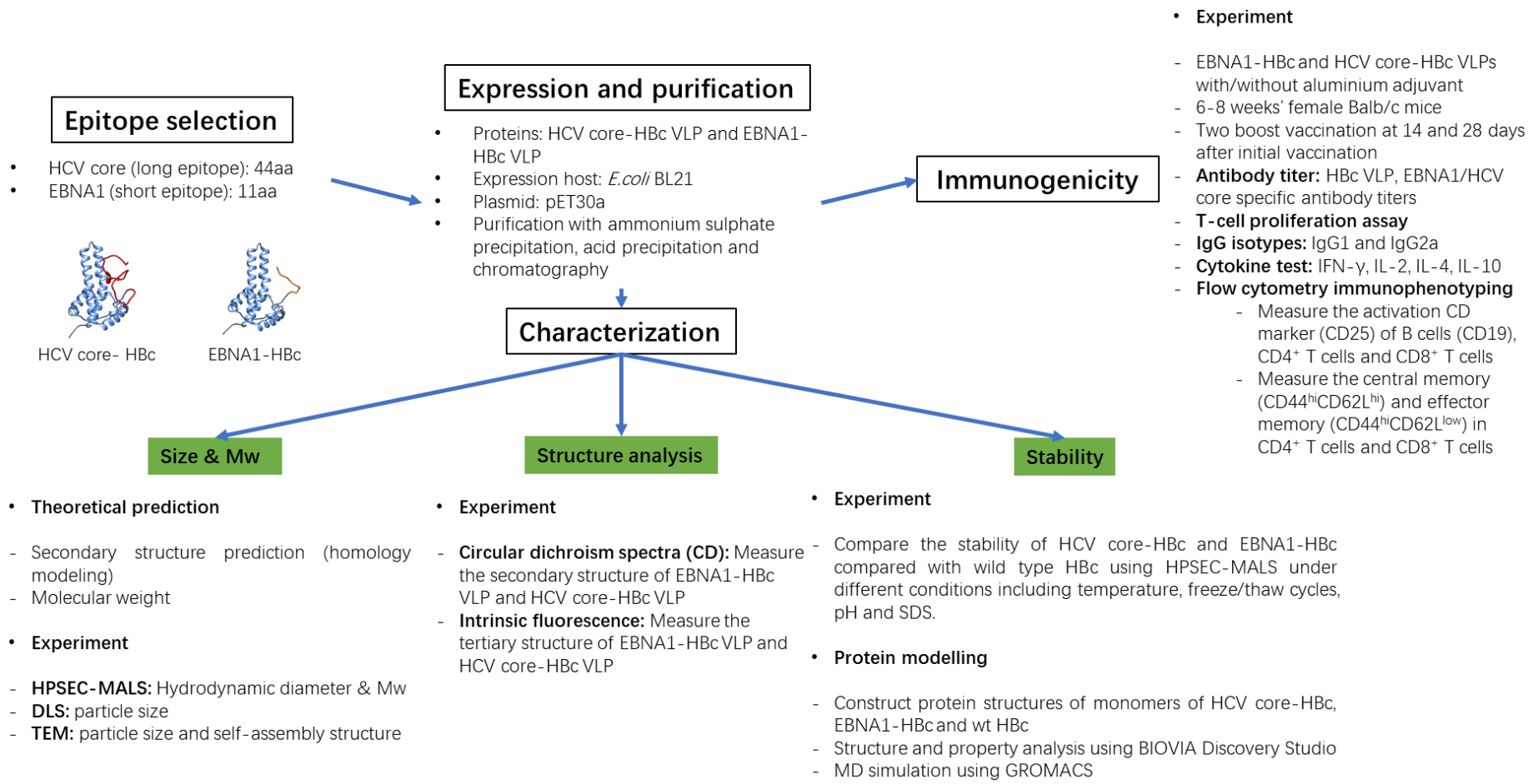

Figure 1.1 The design and experimental structure of the PhD project

1.3 Thesis outline

In Chapter 1, the background, the aim and objectives, structure and thesis outline are introduced.

In Chapter 2, recent development and challenges of VLP-based platform as cancer vaccines were reviewed.

In Chapter 3, chimeric HBc VLPs targeting the EBV and HCV were designed by presenting two foreign epitopes, EBNA1 and HCV core, on the N-terminus of HBc VLP, respectively. Optimizations for the conditions of expression and downstream purification were performed aiming to produce the designed chimeric HBc VLPs with high production yields in soluble form.

In Chapter 4, characterization and the abilities of chimeric EBNA1-HBc VLP and HCV core-HBc VLP to maintain the assembled VLP structure were evaluated under different physical and chemical stresses compared with wild type (wt) HBc VLP to examine the impact of insertion of foreign epitopes on the stability of chimeric HBc VLPs after insertion of different types of epitopes. Protein computational modelling and MD simulation were applied to assist the understanding of the change of the physical property of chimeric VLPs which causes the differences between the stability of wt HBc VLP and chimeric HBc VLPs.

In Chapter 5, chimeric HBc VLP presenting short and non-structural epitope, EBNA1, was constructed and produced using optimized production conditions. *In vivo* humoral and cellular immune response induced by adjuvant free EBNA1-HBc VLP was examined compared with that induced by EBNA1-HBc VLP with aluminium adjuvant.

Long-term protection ability of adjuvant free EBNA1-HBc VLP was evaluated by detection of EBNA1 specific memory T cells produced in the immunized mice.

In Chapter 6, chimeric HBc VLP presenting long and structural epitope, HCV core, was constructed and produced using optimized production conditions. *In vivo* HCV core specific humoral and cellular immune response induced by adjuvant free chimeric HCV core-HBc VLP was examined and compared with chimeric EBNA1-HBc. Then the difference in immunogenicity and the impact on the immune response type caused by insertion of different types of foreign epitopes were evaluated. Long-term immunogenicity of adjuvant free HCV core-HBc VLP was evaluated by detection of HCV core specific memory T cells produced in the immunized mice.

In Chapter 7, the conclusion of the thesis and the future work plan were presented.

References

[1] A.S. Clem, Fundamentals of vaccine immunology, J. Glob. Infect. Dis. 3(1) (2011) 73-78.

[2] K.L. Hartshorn, M.R. White, E.C. Crouch, Contributions of the N-and C-terminal domains of surfactant protein d to the binding, aggregation, and phagocytic uptake of bacteria, Infection and immunity 70(11) (2002) 6129-6139.

[3] J.B. Margolick, R.B. Markham, A.L. Scott, The immune system and host defense against infections, Infectious disease epidemiology: theory and practice (2014) 317-43.

[4] D. Baxter, Active and passive immunity, vaccine types, excipients and licensing, Occup. Med. 57(8) (2007) 552-556.

[5] Centers for Disease Control and Prevention (CDC). https://www.cdc.gov/vaccines/vac-gen/immunity-types.htm. (Accessed April 20 2020).

[6] World Health Organization. https://www.who.int/csr/disease/smallpox/en/.

[7] P. National Research Council Division of Health, P. Disease, Vaccine Supply and Innovation, National Academies Press (US)

Copyright (c) National Academy of Sciences., Washington (DC), 1985.

[8] L. Falzone, S. Salomone, M. Libra, Evolution of Cancer Pharmacological Treatments at the Turn of the Third Millennium, Front. Pharmacol. 9(1300) (2018).

[9] W.F. Bynum, W.F. Bynum, W.F. Bynum, R. Porter, Medicine and the five senses, Cambridge University Press1993.

[10] S. Mukherjee, The emperor of all maladies: a biography of cancer, Simon and Schuster2010.

[11] A. Arnold-Forster, A pathology of progress? Locating the historiography of cancer, BJHS 49(4) (2016) 627-634.

[12] J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012, International journal of cancer 136(5) (2015) E359-E386.

[13] National Cancer Institute (NIH). https://www.cancer.gov/about-cancer/treatment/types. (Accessed March 24 2020).

[14] M. Zapatka, I. Borozan, D.S. Brewer, M. Iskar, A. Grundhoff, M. Alawi, N. Desai, H. Sültmann, H. Moch, M. Alawi, C.S. Cooper, R. Eils, V. Ferretti, P. Lichter, I. Borozan, D.S. Brewer, C.S. Cooper, N. Desai, R. Eils, V. Ferretti, A. Grundhoff, M. Iskar, K. Kleinheinz, P. Lichter, H. Nakagawa, A.I. Ojesina, C.S. Pedamallu, M. Schlesner, X. Su, M. Zapatka, P. Pathogens, P. Consortium, The landscape of viral associations in human cancers, Nat. Genet. 52(3) (2020) 320-330.

[15] S. Kruger, M. Ilmer, S. Kobold, B.L. Cadilha, S. Endres, S. Ormanns, G. Schuebbe, B.W. Renz, J.G. D'Haese, H. Schloesser, V. Heinemann, M. Subklewe, S. Boeck, J. Werner, M. von Bergwelt-Baildon, Advances in cancer immunotherapy 2019 – latest trends, J. Exp. Clin. Cancer Res. 38(1) (2019) 268.

[16] N.K.C. Linda H.L. Lua, Frank Sainsbury, Yap P. Chuan,Nani Wibowo, Anton P.J. Middelberg, Bioengineering Virus-Like Particles as Vaccines, Biotechnology and Bioengineering 111 (2014) 425-440.

[17] Z. Wang, H. Gao, Y. Zhang, G. Liu, G. Niu, X. Chen, Functional ferritin nanoparticles for biomedical applications, Frontiers of chemical science and engineering 11(4) (2017) 633-

646.
[18] J. Liu, L. Miao, J. Sui, Y. Hao, G. Huang, Nanoparticle cancer vaccines: Design considerations and recent advances, Asian Journal of Pharmaceutical Sciences (2019).
[19] P.-L. Lollini, F. Cavallo, P. Nanni, G. Forni, Vaccines for tumour prevention, Nature Reviews Cancer 6(3) (2006) 204-216.
[20] M.O. Mohsen, D.E. Speiser, A. Knuth, M.F. Bachmann, Virus-like particles for vaccination against cancer, Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology n/a(n/a) e1579.
[21] D. Yan, Y.-Q. Wei, H.-C. Guo, S. Sun, The application of virus-like particles as vaccines and biological vehicles, Applied microbiology and biotechnology 99 (2015).
[22] H.K. Ong, W.S. Tan, K.L. Ho, Virus like particles as a platform for cancer vaccine development, PeerJ 5 (2017) 4053-4053.
[23] C. Lemke, A. Salem, A. Krieg, G. Weiner, Abstract 1417: Combination cancer immunotherapy using checkpoint blockade and intratumoral virus-like particles containing CpG ODN, Cancer Res. 76(14 Supplement) (2016) 1417.
[24] L. Klimek, T. Kündig, M.F. Kramer, S. Guethoff, E. Jensen-Jarolim, C.B. Schmidt-Weber, O. Palomares, M.O. Mohsen, T. Jakob, M. Bachmann, Virus-like particles (VLP) in prophylaxis and immunotherapy of allergic diseases, Allergo Journal International 27(8) (2018) 245-255.
[25] S.A. Keller, M. Bauer, V. Manolova, S. Muntwiler, P. Saudan, M.F. Bachmann, Cutting Edge: Limited Specialization of Dendritic Cell Subsets for MHC Class II-Associated Presentation of Viral Particles, The Journal of Immunology 184(1) (2010) 26.
[26] A.F. Sander, P.-L. Lollini, Virus-like antigen display for cancer vaccine development, what is the potential?, Expert Review of Vaccines 17(4) (2018) 285-288.
[27] J.d.S. Apostólico, V.A.S. Lunardelli, F.C. Coirada, S.B. Boscardin, D.S. Rosa, Adjuvants: Classification, Modus Operandi, and Licensing, Journal of Immunology Research 2016 (2016) 1459394.
[28] J. Chroboczek, I. Szurgot, E. Szolajska, Virus-like particles as vaccine, Acta Biochim Pol 61(3) (2014) 531-9.
[29] R. Noad, P. Roy, Virus-like particles as immunogens, Trends Microbiol. 11(9) (2003) 438-44.
[30] A.P. Middelberg, T. Rivera-Hernandez, N. Wibowo, L.H. Lua, Y. Fan, G. Magor, C. Chang, Y.P. Chuan, M.F. Good, M.R. Batzloff, A microbial platform for rapid and low-cost virus-like particle and capsomere vaccines, Vaccine 29(41) (2011) 7154-62.
[31] M.W. Liew, A. Rajendran, A.P. Middelberg, Microbial production of virus-like particle vaccine protein at gram-per-litre levels, J Biotechnol 150(2) (2010) 224-31.

Chapter 2: Literature Review: Recent technology and challenges of virus like particle (VLP)-based platform applied in cancer vaccines development

2.1 Introduction

Recent researchers have proven that viruses are associated with various types of cancers globally [1]. These viruses are called oncoviruses which mainly include human papillomavirus (HPV), hepatitis B virus (HBV) and hepatitis C virus (HCV), Epstein–Barr virus (EBV), Merkel cell polyomavirus, Kaposi's sarcoma-associated herpesvirus (KSHV) and human T-lymphotropic virus [2]. To develop the treatments against these oncoviruses, immunotherapy, a novel approach to treat cancers by using patients' own immune system [3], has been investigated and developed. It is believed that immunotherapy is an efficient approach to treat diseases of hematological cancers and virus leading cancers. There are several types of immunotherapy that have been developed against cancers including immune checkpoint inhibitors, T-cell transfer therapy, monoclonal antibodies, immune system modulators and cancer vaccines [4]. Among them, cancer vaccines are regarded as one of the best candidates [5] because of their exquisite specificity, low toxicity, and the potential for a durable treatment effect due to immunologic memory [6].

In the investigation of effective cancer vaccines, one of the subunit vaccines, virus-like particle (VLP), has attracted researchers' attention because of its unique structure and its merits. VLPs are molecules with structures similar to natural viruses and are mainly constructed with capsid proteins of viruses [7]. However, VLPs have no viral genes, which means that they are non-infective and non-replicative. These properties make VLPs promising candidates for the development of effective and safe cancer vaccines. However, only a few VLP-based cancer vaccines are available in the market at present such as HBV, HPV and Influenza vaccines [8-11].

To produce VLP-based cancer vaccines, challenges could be much more than the production for traditional vaccines for the following reasons: 1) cost-effective bioprocess development to produce designer VLP vaccines, 2) complexity to maintain the biomolecular structure of VLP vaccines, 3) *in vivo* immunogenicity related directly to the molecular structure of VLP vaccines [12].

To better understand the development and overcome the challenges in the production of VLPs as cancer vaccines for immunotherapy, related literatures are reviewed in this chapter. In this chapter, seven parts are included. 1) vaccine types are described to understand the properties and features of different vaccines, 2) different types of cancer vaccines are reviewed to understand the development of recent cancer vaccines, 3) oncovirus is reviewed to understand the application of immunotherapy in cancer treatment, 4) studies on chimeric VLPs targeting oncoviruses are reviewed to understand the development of recent chimeric VLP vaccines, 5) computational protein

modelling of VLPs is reviewed to assist the study on the biomolecule's design and stability. 6) production process of chimeric VLP-based vaccines is reviewed to fully understand the bottlenecks of expression and purification of chimeric VLP vaccines, and 7) characterization methods for VLPs are reviewed to understand the recent technology for analysis of the structure and properties of VLPs.

2.2 Vaccine types

Immunity can be acquired through two ways which are the passive or the active means [13]. Active immunity is acquired when the immune system is exposed to an antigen. The immune system takes some time to response but normally the acquired immunity can last longer time [14]. In comparison, passive immunity is acquired by giving the specific antibody against the infection. The response to passive immunity is normally immediate but the immune efficiency lasts shorter time compared with active immunity [14]. Both passive and active immunization can be acquired from natural or artificial sources.

In the modern immunotherapy for diseases, patients are exposed with a pathogenic antigen which can trigger the immune response of patients by active immunization to develop immunity to specific antigens [15]. In this way, patients after vaccination can obtain long-term immunity against the pathogenic antigen because of the activation of immune system. Excellent examples of immunotherapy through active immunization are the influenza vaccination and smallpox vaccination [16].

There are several different processes to make vaccines. Traditionally, vaccines were

made from 1) live attenuated viruses through different methods to weaken its infectious and 2) inactivated viruses which are killed to destroy producing capacity of viruses [17]. However, the safety issues for inactivated vaccines and live attenuated vaccines limit their development and applications. To reduce the risk during the vaccination, advanced vaccines such as mRNA vaccines and subunit vaccines are then developed. **Table 2.1** summarizes the different types of vaccines and their commercial vaccines.

Table 2.1 Vaccine types and their available vaccines (modified from [18])

Vaccine type	Available vaccines
Traditional vaccines	
Live attenuated virus	Measles, mumps, rubella (MMR combined vaccine); Varicella (chickenpox); Influenza (nasal spray); Rotavirus; Zoster (shingles); Yellow fever
Inactivated virus	Polio (IPV); Seasonal influenza; Hepatitis A virus (HAV); Rabies
Advanced vaccines	
mRNA vaccines	COVID-19, HIV-1
Subunit vaccines	Influenza (injection); Haemophilus influenza type b (Hib); Pertussis (part of DTaP combined immunization); Pneumococcal; Meningococcal, Human papillomavirus (HPV); Hepatitis B virus (HBV)

2.2.1 Traditional vaccines

2.2.1.1 Live attenuated vaccines

As shown in **Table 2.1** live attenuated virus vaccines and inactivated virus vaccines are

classified as traditional vaccines. Various techniques have been applied to produce traditional vaccines. One traditional approach to produce live attenuated vaccines is passing target virus through a series of cell cultures or animal embryos (typically chick embryos) to weaken its infection and adaptation of the virus to the growth in the cultured cells. This process is accompanied by a gradual loss of virulence of target virus for the natural host [19, 20]. For example, in the chicken embryos culture process, the virus is grown in a series of embryos to increase its ability to replicate in chicken cells and to reduce its ability to replicate in human cells. In theory, through hundreds of passes, the attenuated virus gradually loses its replicability in human cells, and its infectivity against human becomes significantly weaken to render it safe for human vaccination. Therefore, attenuated virus retains the ability to trigger the immune response against the virus and protect human from future infection [21]. However, in practical application, this method remains the risk that the virus can recover its replication ability after entering human and may cause the disease. In addition, mutations of virus could happen when the attenuated virus replicates in the human body, and it could turn to be a more deadly form. Oral polio vaccine (OPV) is one example. OPV is a kind of live attenuated vaccines when it was developed for the treatment of poliomyelitis. It is reported that the attenuated polio virus could turn to a virulent form and cause some rare cases of paralytic polio[22]. Therefore, OPV is banned for use in the United States and has been replaced with the inactivated vaccine.

2.2.1.2 Inactivated vaccines

Inactivated vaccine is another popular traditional vaccine. The viruses are replicated by approaches such as cell cultivation or chicken embryos cultivation. The produced viruses are then inactivated by heat or chemicals such as formaldehyde or formalin [23]. The process destroys the replication ability of the pathogen, while keeps its antigenicity to be recognized by human body and trigger the immune response against the target pathogen [24].

Activated vaccines show much lower risks of reverting the pathogen to a more virulent form since inactivated pathogens lose its replication ability completely [23]. However, the protection period of inactivated is normally shorter compared with live attenuated vaccines. To obtain the similar long-term immunogenicity, several boosters need to be used. Inactivated polio vaccine and the seasonal influenza vaccine are two common inactivated vaccine in the market at present as shown in **Table 2.1** [18].

2.2.2 Advanced vaccines

2.2.2.1 Messenger RNA (mRNA) vaccines

mRNA has been investigated as an advanced therapeutic tool in the fields of vaccine development. mRNA vaccines can be produced through *in vitro* transcription of a complementary DNA (cDNA) template using a bacteriophage RNA polymerase [25]. The application of mRNA vaccine has several merits over traditional vaccine candidates as well as DNA-based vaccines [26]. Firstly, mRNA is a non-infectious platform. In this case, it is safer compared with traditional vaccines with potential risk

of infection or insertional mutagenesis. Secondly, several approaches for the modifications of mRNA have been developed to increase its stability and translatability [27, 28]. mRNA carriers such as polymers and nanoparticles have been developed to enable the rapid uptake and expression of mRNA vaccines in the cytoplasm [29]. Finally, rapid, inexpensive and scalable production of mRNA vaccines have been developed thanks to the high yields of *in vitro* transcription reactions. However, mRNA can be degraded during normal cellular processes. This could affect *in vivo* half-life of mRNA vaccine which influences on its immunogenicity efficacy. To overcome this issue, various modifications and delivery methods including covalent conjugates, protamine complexes, nanoparticles based on lipids or polymers, and hybrid formulations have been investigated [29, 30]. One recent application of mRNA vaccine is the novel COVID-19 vaccine manufactured by Pfizer-BioNTech and Morderna. Moderna reported that vaccine candidate mRNA-1273 achieved an efficacy of 94.1% during the phase 3 clinical trial, enrolled 30,420 volunteers who were randomly assigned in a 1:1 ratio to receive either vaccine or placebo (15,210 participants in each group) [31]. Another Pfizer-BioNTech vaccine candidate, BNT162b2, was 95% effective in preventing COVID-19 (95% credible interval, 90.3 to 97.6) during the phase 3 clinical trial, which enrolled 43,548 participants [32].

2.2.2.2 Subunit vaccines

As shown in **Table 2.1**, subunit vaccine contains only part or parts of the pathogens that can induce protective immune response, and it attracts the attention of researchers

in the last few decades [33-35]. Since subunit vaccines use parts of the pathogens to retain the ability to trigger a response from the immune system, it is much safer in the application compared with traditional vaccines. One specific part from the pathogen which is suitable for presenting as the antigen is isolated to form subunit vaccine. The subunit vaccine technique is now commonly applied in the development of acellular pertussis and influenza vaccines [21]. As is listed in **Table 2.2**, four different types of subunit vaccines including protein subunit, peptide subunit, polysaccharide subunit and virus like particle (VLP) based vaccines have been developed.

Subunit vaccines can be generated by using genetic engineering such as recombinant protein technique [36] or conjugating polysaccharide to carrier proteins [37]. Recombinant vaccines containing specific antigens targeting different diseases were developed. By fusing the gene sequence of the antigen with other protein carrier sequence, the recombinant vaccines can be recognized by human body and can trigger antigen specific immune response in the body for further protection against the pathogens [35, 38, 39]. Subunit vaccines obtain several advantages over traditional vaccines in terms of safety, short production and specific immune target [40]. However, in the development of subunit vaccines, the major challenges are the low immunogenicity efficiency and the stability of the vaccine candidates [33]. Further modification and development of novel subunit vaccines are required.

Table 2.2 List of different types of subunit vaccines.

Type	Target disease or virus
Protein subunit	SARS-CoV-2
Peptide subunit	Malaria Parasite, Alzheimer's Disease, Pneumococcal Disease, HCV, Influenza Virus and HIV-1
Polysaccharide subunit	Pneumococcal Disease
Virus like particle	HBV, HPV, HEV, HAV and Influenza A

2.2.2.2.1 Protein-based subunit vaccines

Figure 2.1 illustrates the structure of classical vaccine with whole pathogen, protein-based subunit vaccine and peptide-based subunit vaccine. Protein-based subunit vaccine (**Figure 2.1B**) is formed by assembly of a single protein molecule with other protein molecules to form a complex [41]. Protein-based subunit vaccine can be produced by isolation of a specific protein from a virus or expressed as a recombinant protein subunit using different expression system such as bacteria and yeast. However, protein-based vaccines have challenges including the vaccine efficacy, the cost of recombinant protein production, stability and purity problems. The use of a whole foreign protein may cause autoimmune responses. For example, M protein from group A streptococcal (GAS) is the major virulence factor of GAS and a target for vaccine development [42]. However, the conserved C-terminal regions of M protein could elicit autoimmunity, therefore the whole protein cannot be used as an antigen without substantial risk of toxic side-effects [43]. Recently, Spike protein of SARS-CoV-2 has been selected as the candidate to develop protein-based subunit vaccine against

COVID-19. S-Trimer COVID-19 vaccine (SCB-2019) has been developed using Trimer-Tag to allow for trimerization of SARS-CoV-2 spike (S)-protein [44]. In a phase 1 clinical trial, SCB-2019 was confirmed to induce high level of antibodies against SARS-CoV-2. In addition, strong Th1-biased cell-mediated immune responses were observed. Further phase 2 and 3 clinical trial is needed to confirm the vaccine efficacy and stability of this novel protein-based subunit vaccine against SARS-CoV-2.

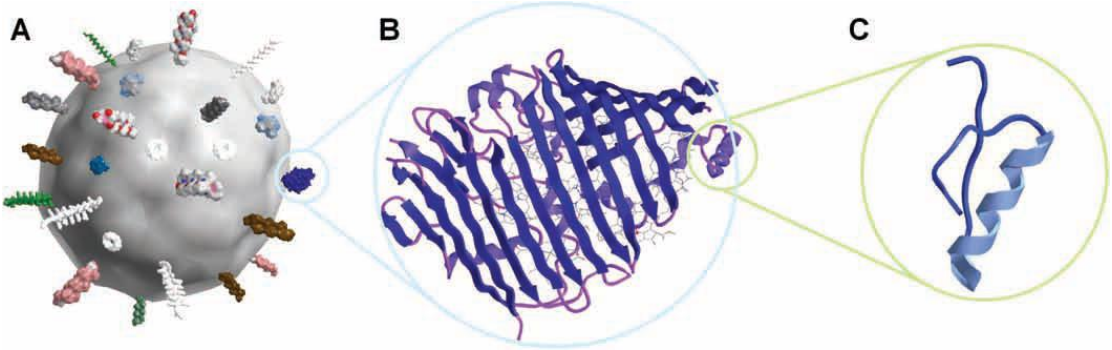

Figure 2.1 Schematic evolution of vaccines: (A) classical vaccine incorporating whole pathogen, (B) protein-based subunit vaccine, and (C) peptide-based subunit vaccine [45].

2.2.2.2.2 Peptide-based subunit vaccines

This autoimmune response can be reduced or eliminated by using short peptide as epitope rather than large pathogen components as is shown in **Figure 2.1C**. Peptide-based vaccines can be produced with high purity and well-defined composition using low-cost expression system such as bacteria and yeast [45]. The produced peptide-based subunit vaccines are more stable, water-soluble than protein-based subunit vaccines. In addition, several peptide epitopes including different subtypes of a pathogen, different stages in the life cycle of a pathogen or even epitopes from multiple pathogens can be loaded to one peptide-based subunit vaccine [45]. Peptide-based

subunit vaccine candidates are expected to induce strong immune response mediated by lymphocytes such as cytotoxic T-lymphocytes (CTL, CD8+ T-cells) and B-cells [46]. However, peptide epitopes are very susceptible to enzymatic degradation in the body, which is significantly influence on the vaccine efficacy of peptide-based subunit vaccines. To enhance the metabolic stability of a peptide epitope, various approaches have been developed including modification the C- or N-terminal of the peptide subunit, synthesis in a retro-inverted form and conjugate with carriers [47-49]. Peptide-based subunit vaccines have been adapted in the development of vaccines against viruses such as HCV, Influenza Virus and HIV-1 and diseases such as Malaria Parasite, Alzheimer's Disease, Pneumococcal Disease [45]. However, the major challenge for peptide vaccines is the weaker overall immune response in comparison to traditional vaccines While this issue is of particular relevance in infectious diseases, it may also pertain to other disease areas as well, because stronger immune responses can often be associated with greater protection and durability.

2.2.2.2.3 Polysaccharide-based subunit vaccines

Capsular polysaccharides (CPS) from bacteria have been investigated and employed for the production of polysaccharide-based subunit vaccines. Pneumococcal disease is one target disease for the development of polysaccharide-based subunit vaccines. The latest pneumococcal conjugate vaccine contains 13-valent pneumococcal serotypes (PCV13). This vaccine's efficacy of PCV13 against pneumococcal infections was 45.6% (95% confidence interval [CI] = 21.8%–62.5%) [50]. However, two major issues were

observed in the development of pneumococcal polysaccharide-based subunit vaccines. One issue is that the pneumococcal polysaccharide-based subunit vaccines are not effective in infants and young children (under 18–24 months) [51]. In theory, the repeating subunits of pneumococcal polysaccharide-based subunit vaccines can stimulate immune response in B cells independent of T-cell. However, the ability to mount a T-cell–independent immune response of infants and young children is weak [52]. Another issue is that the immune response induced by polysaccharide-based subunit vaccine is short term immunity and revaccination is needed [51]. In this case, modification and redesign of polysaccharide-based subunit vaccines are required for its application as promising vaccine candidates.

2.2.2.2.4 Virus like particle (VLP)-based vaccines

VLP is a special form of protein-based subunit vaccine and is composed of the capsid proteins of virus and it obtains the molecular structure similar to the virus while has no viral gene sequence. VLPs have the morphology of a highly repetitive immunogenic surface structure which enables VLP to induce strong immune response in body. Since VLPs have no viral genomes, they are unable to infect or replicate in the body. This non-infectious feature of VLP proteins greatly improves their safety compared with other subunit vaccine candidates [53, 54] .

Briefly, VLPs can be mainly categorized as non-enveloped and enveloped structures. Non-enveloped VLPs were assembled from single viral subunit expressed by different expression systems such as mammalian cells, insect cells, yeasts, bacteria [10]; while

the assembly process of enveloped VLPs needs the assistance of lipid membranes from expression cells [55]. Non-enveloped VLPs are generally easier in the production and purification compared with enveloped VLPs, while enveloped VLPs are more flexible and can be integrated with different pathogens [56]. In this case, enveloped VLPs are more suitable to present full-length monomeric or multimeric conformational antigens from target viruses and non-enveloped VLPs are more suitable for presenting short epitope from target viruses [57].

Due to the unique structure of VLPs, they have been broadly investigated and developed as preventive cancer vaccines for the treatment of different cancers such as cervical cancers and Hepatocellular carcinoma. In addition, VLPs are also applied as preventive vaccines in the treatment of other infectious diseases such as influenza and foot-mouth disease. VLPs are in the structure of highly repeated order, and such assembled structure normally are considered as effectively crosslinked B-cell receptors [58]. It is reported that VLPs can trigger the strong immune response with the assistance of the antigen presenting cells due to their unique structure. Due to the characteristic of VLPs, VLPs show good self-adjuvating property and different VLP-based vaccines constructed with the structure proteins from the virus have been developed after the first recombinant Hepatitis B surface antigen (HBsAg) VLP was successfully commercialized in 1982 [12]. In addition to the approach to use the structure proteins from cancer related virus to form the VLPs as vaccine candidates, VLPs were also employed as an efficient delivery platform for tumour antigens by genetic engineering

or conjugation. A successful vaccination depends largely on its immunogenicity and presentation efficiency of the tumour antigens, and VLPs obtain many advantages over other candidates including the safety as well as the efficiency [53, 54]. However, up to date, few VLP-based vaccines against viruses including human papilloma virus (HPV), hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis E virus (HEV), and influenza A have been commercialized (as listed in **Table 2.3**). Among them, HPV VLP and HBV VLP are reviewed in the following session.

Table 2.3 Current commercial VLP-based vaccines [59]

Virus Species	Antigen/s	Adjuvant	Commercial Name (Company)
HPV	Capsid protein L1	AS04	Cervarix (GSK)
HPV	Capsid protein L1	Aluminium	Gardasil (Merck)
HBV	HBsAg	Aluminium	Engerix-B (GSK)
HBV	HBsAg	Aluminium	Recombivax HB (Merck)
HBV	HBsAg	Aluminium	GenHevac B (Pasteur)
HBV	HBsAg	AS04	Fendrix (GSK)
HEV	Capsid protein CP	Aluminium	Hecolin (Xiamen Innovax Biotech)
Influenza A	HA and NA glycoproteins	Liposome	Inflexal V (Crucell)
HAV	Inactivated HAV	Virosome	Epaxal (Crucell)

2.2.2.2.4.1 Human papillomavirus (HPV) vaccines

Cervical cancer is reported to be a server cancer and its survival rate varies depending on the stage [60]. HPV is regarded as the main agent to cause the cervical cancer, and it was reported that HPV was found positive in nearly 100 % patients such as different

types of HPV (HPV-16 and HPV-18) [60]. At present, the most promising way to reduce the risk of cervical cancer is the early vaccination of HPV vaccines as preventive protection. Multivalent Gardasil, Cervarix, and Gardasil 9 are the commercial HPV vaccines available in the market. L1 protein of HPV which is the major capsid protein of HPV can self-assembly into VLPs and is used to generate preventive vaccines. The commercial Gardasil 9 vaccine provides fully vaccinated people with protection against nine types of HPV including: 1) types 16 and 18, the two types that cause the majority of HPV-related cancers, 2) the five next most common HPV types associated with cervical cancer (types 31, 33, 45, 52 and 58) and 3) two non-cancer-causing HPV types (types 6 and 11), which cause 90% of genital warts [61]. Research studies have shown early signs of the vaccine's success including: 1) a 77% reduction in HPV types responsible for almost 75% of cervical cancer, 2) almost 50% reduction in the incidence of high-grade cervical abnormalities in Victorian girls under 18 years of age and 3) a 90% reduction in genital warts in heterosexual men and women under 21 years of age [62].

Researchers have found that in addition to L1 major capsid protein of HPV, L2 minor capsid protein of HPV with a highly conserved structure could be another option for the development of novel HPV VLP-based vaccines [63]. It is reported that vaccination with L2 peptide can induce cross-neutralizing antibodies and conferred cross-protection *in vivo*. However, the induced antibody titer was much lower compared with the vaccines prepared using L1 capsid protein [64, 65]. Following this lead, a novel HPV

VLP-based vaccine was generated by fusing L2 sequence to the C-terminal of L1 protein to improve its immunogenicity and induced the high antibody titer [66]. *Nicotiana benthamiana* was used as the host cell to express the novel recombinant L1/L2 VLP. The molecular characterization indicated that L2 was exposed on the surface of expressed chimeric VLP. The immunogenicity evaluation confirmed the improved humoral immune response against both L1 and L2 antigen and the novel chimeric VLP vaccine were demonstrated to protect the mice from HPV-16 and HPV-52 [66]. Similarly, Huber et al. (2015) constructed the HPV VLP vaccine by fusing the L2 protein from HPV-45 to the L1 protein from HPV-18. The *in vivo* immunization results indicated that the chimeric L1/L2 HPV vaccine can cross-protect against four different types of HPVs after mice were injected with antisera from the L1/L2 HPV VLP vaccine immunized rabbits [67]. Also, there is no available therapeutic HPV vaccines to treat pre-existing cervical cancers. To fill the gap, researchers have made attempts to produce a chimeric therapeutic vaccine by inserting the non-structural E7 protein of HPV into infectious bursal disease virus to form VLP-based vaccine (E7 VLP vaccine). E7 protein is proven to be a tumour-specific antigen, and is over expressed in the patients with HPV-associated cervical cancers [68]. The preliminary results from the tumour challenge experiment indicated that tumour of mice vaccinated with E7 VLP vaccine was completely inhibited [69]. Further research on this approach needs to be explored before clinical trial and marketing. The lessons and experience gained from developing HPV vaccines have inspired researchers in developing other similar VLP-

based vaccines for treatments of other cancers.

2.2.2.2.4.2 Hepatitis B virus (HBV) vaccines

HBV related liver cancers are diagnosed in large number every year and its death rate is high among all cancers [70] and the survival rate of liver cancer patients is only about 25-45% [71]. Around 30 % of the world population are infected with HBV, and among them, around 350 million people developed chronic infection [72]. It is reported that around 50% of liver cancers are related with the infection of Hepatitis B virus or Hepatitis C virus [73]. To date, commercial HBV preventive vaccines is available in the market. It has been proven that preventive vaccination is the most effective way to reduce the risk of liver cancers [74]. However, the limitation to access these vaccines and lack of therapeutic HBV vaccines have inhibited the step to eradicate HBV infection rate and reduce the risk of liver cancer.

After the first recombinant hepatitis B vaccine was licensed in the United States in July 1986, many optimized HBV vaccines were produced [75]. There are several HBV VLP vaccines on the market currently including Engerix-B, Enivac HB, Gene Vac-B, Hepavax-Gene, and Recombivax HB as shown in **Table 2.3** [7].

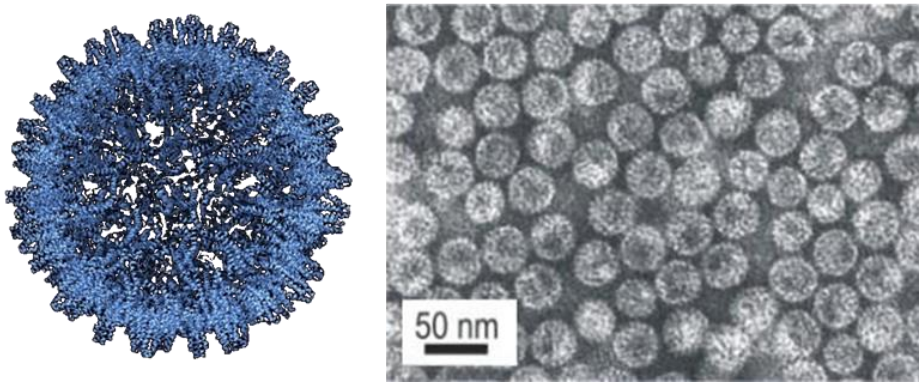

Figure 2.2 Structure of Hepatitis B virus core VLPs [76]

Figure 2.2 shows the structure of Hepatitis B core (HBc) VLPs. HBc VLP is composed of the core capsid protein from HBV and the HBc VLP has two major isomorphs with triangulation numbers T = 4 and 3 that consist of 240 and 180 HBc monomers [77]. The sizes of wild type (wt) HBc VLP in two isomorphs are 35 and 32 nm in diameter, respectively. HBc VLPs have been reported to be successfully applied in the presentation of different foreign antigens. For example, Ye et al. (2014) successfully constructed the chimeric SP55-HBc and SP70-HBc VLP proteins to present the SP55 and SP70 peptides from Enterovirus 71 (EV71) using *Escherichia coli* (*E. coli*) expression system and the produced chimeric VLP vaccine induced epitope-specific and HBc specific antibody responses in mice [78]. However, as claimed in the report, the chimeric SP55-HBc and SP70-HBc VLP proteins were expressed highly in the inclusion bodies, which potentially increased the complexity in production process such as protein refolding and assembly process. More applications of HBc VLP in the production of chimeric VLP-based vaccines are described in **section 2.5**.

2.3 Cancer vaccines

Cancer vaccines play an important role in treating infections which lead to cancers and other diseases by inducing strong and long term self-immune response [5]. As shown in **Figure 2.3**, after immunization, cancer vaccines stimulate dendritic cell (DC) and trigger Class I major histocompatibility complex (MHC) pathway for cellular immune response and Class II MHC pathway for humoral immune response. Cancer vaccines have two major categories: preventive cancer vaccine and therapeutic cancer vaccine

[79]. Preventive cancer vaccine aims to induce the immune response of the body to defend the viral infection which can consequently cause cancers; while therapeutic cancer vaccine aims to stimulate the immune system of the patients to kill or inhibit the growth of tumour cells [79].

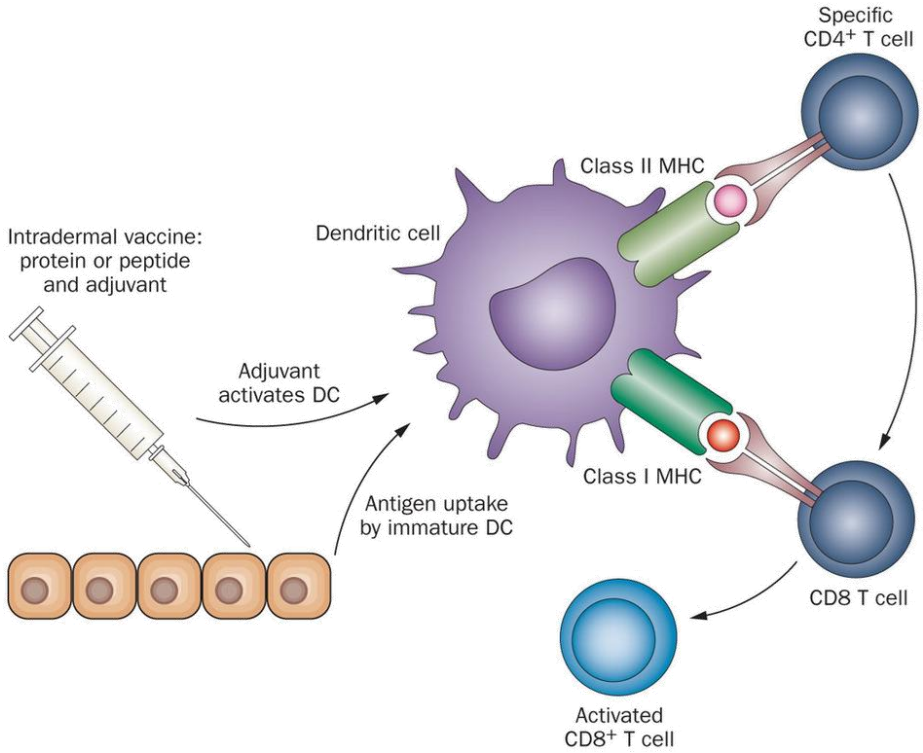

Figure 2.3 Mechanism of cancer vaccines [80]. DC: dendritic cell, MHC: major histocompatibility complex

2.3.1 Therapeutic cancer vaccines

Therapeutic cancer vaccines are designed to induce strong immune response of the host against the tumour cells leading to reduce or control the growth of the tumour. However, during the development of efficient therapeutic cancer vaccines several barriers remain. Among them, tumour-induced immunosuppression in tumour microenvironment (TME) is regarded as the major factor limiting the development of therapeutic cancer vaccines [81]. During the study of therapeutic cancer vaccines, a few immunosuppressive

leukocytes, including regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) were found in TME. These leukocytes generate cytokines, such as TGF-β and IL-10, that enhance the tumour growth, metastasis, and angiogenesis [82]. In addition, tumour-associated macrophage (TAMs) also influences the activation of T-cells and sometimes causes the death of activated T-cells [83, 84]. In this way, the efficiency of the immunotherapy to the tumour is largely reduced. Tegerstedt et al. (2005) have developed a VLP-based breast cancer vaccine by fusing the antigen, TAA HER-2/neu (Her2), to murine polyomavirus VLPs to produce a chimeric therapeutic cancer vaccine [85]. Her2 was selected as the antigen because it is recognized as the primary biomarker for the breast cancer which are overexpressed during the breast tumour's growth [86]. They found that the chimeric Her2-Py VLP was able to induce strong Her2 specific T-cell immune response *in vivo*. This also leads to the tumour reduction and longer surviving time when examined by *in vivo* tumour challenge experiment. However, humoral immunity was barely detected. This could result from the position for the insertion of the antigen as Her2 antigen was buried internally in the vaccine. Another issue in the development of these breast cancer therapeutic vaccines is that the vaccine efficacy depends on the immunization timing, probably due to the existence of TME. In Tegerstedt's study, delayed immunization only postponed tumour outgrowth but had a minor effect on the protection of breast cancer. Therefore, a successful therapeutic cancer vaccine should be able to trigger immune response against tumour cells and avoid the effect of TME immunosuppressive state.

2.3.2 Preventive cancer vaccines

Preventive cancer vaccines are designed to induce the immune response of the host against the origins that lead to the cancers. The origin which has been broadly studied is the oncovirus. Several oncoviruses including HBV, HCV, HPV, EBV, Kaposi's sarcoma-associated herpesvirus, human T-cell lymphotropic virus, and merkel cell polyomavirus have been recognized as the triggers for different cancers, and the preventive cancer vaccines against these viruses have been broadly investigated [87]. Preventive vaccines against these viruses have been developed and investigated. One of the most successful samples is the HPV vaccine which prevents the cervical cancer. Huber et al. (2015) developed a preventive vaccine, 18L1-45RG1, by fusing the L2 protein of HPV-45 to the L1 protein of HPV-18. In the study, 18L1-45RG1 showed effective protection of mice against the infection of different types of HPVs after the mice were injected with serum from immunized rabbits with preventive 18L1-45RG1 vaccine [67]. With the proven results of HPV vaccine against a broad range of HPVs, it is now commercially available world-widely. However, for the immunotherapy treatment of cervical cancer, only preventive HPV vaccine was commercially available and there is no therapeutic vaccine for cervical cancer yet.

2.4 Oncovirus

In **Table 2.4**, the information regarding the viruses and their related cancers are summarized. HBV, HCV, HPV, EBV and Human T-cell leukaemia virus type 1 (HTLV-1) are five top viruses that can lead to cancers through chronic inflammation

and direct carcinogens act. Comparing with traditional approaches for cancer treatment including invasive surgeries, chemotherapy, and radiotherapy, cancer vaccine treatment does less harm to the patients' body and cause less side effects which are the two major issues in traditional cancer treatment approaches [88]. Among all investigated oncoviruses, HBV and HPV are two widely studied viruses and some commercial vaccines against these two viruses have been developed (listed in **section 2.2.2.2.4**). However, there is no commercial vaccines against the other two major oncoviruses, EBV and HCV, which are also related to severe cancers, even though HCV has been identified as the cause of severe liver disease and liver cancers by Harvey J. Alter, Charles M. Rice and Michael Houghton who awarded the 2020's Nobel Prize in Physiology or Medicine.

Table 2.4 Oncoviruses and related cancers (modified from [7])

Virus	Oncoviruses associated cancers	Mechanism of Carcinogenesis
HBV	Hepatocellular carcinoma	Chronic inflammation
HCV	Hepatocellular carcinoma	Chronic inflammation
HPV	Cervical, vulva, vagina, penis, anus and oropharynx cancers.	Direct carcinogens act via expression of viral oncoproteins
EBV	Burkitt's lymphoma, Hodgkin lymphoma, B cell lymphoma, nasopharyngeal carcinoma, gastric and sporadic carcinoma.	Direct carcinogens act via expression of viral oncoproteins
HTLV-1	Adult T-cell leukemia	Direct carcinogens act via expression of viral oncoproteins

2.4.1 Epstein-Barr virus (EBV)

EBV is an oncogenic γ-herpesvirus and is considered to be related to various cancers

in human populations [89]. **Figure 2.4** illustrates the structure of EBV. EBV is a double-stranded DNA virus with enveloped structure. It is composed of envelop protein, envelope layer, viral tegument nucleocapsid layer and viral genome and the size of EBV is about 122–180 nm in diameter [90]. EBV is associated with Burkitt lymphoma [91]. Infection by EBV is often asymptomatic, however, EBV infection can turn pathologies from infectious mononucleosis (IM) to severe cancers. It is found that a preventive vaccine against EBV is able to prevent IM, and thus, potentially decrease risk of cancers [92]. In addition to IM, researchers have found that EBV is involved in the development of several autoimmune diseases such as multiple sclerosis [93]. The main infection pathway for EBV is saliva. EBV virions mainly target epithelial cells and B cells after entering the hosts [94]. Preventive and therapeutic vaccines against EBV are needed to prevent the EBV infection and controlling EBV-associated diseases such as cancers.

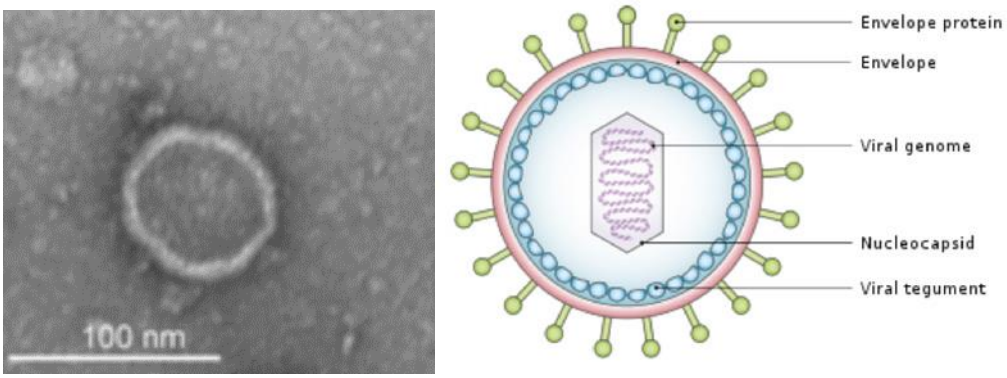

Figure 2.4 TEM image and schematic representations of EBV [95]

It is reported that Burkitt's lymphoma, Hodgkin lymphoma, B cell lymphoma, nasopharyngeal carcinoma, gastric and sporadic carcinoma are related to the infection of EBV [96, 97]. EBV-VLP composed of capsid protein of EBV is a promising candidate for the preventive vaccine. Ruiss et al. (2011) engineered EBV genome to

remove all potential viral oncogenes while maintain the sequence for the assembly of viral capsid to form the EBV VLP. The engineered EBV VLP protein was expressed using 293-VII+ cell lines. Produced EBV-VLPs can both induce both humoral and cellular immune response [98]. EBV-specific $CD8^+$ T cells activation was detected in the mice immunized with EBV VLPs. Recently, one novel chimeric Newcastle disease virus (NDV) VLPs were constructed to present the heterologous antigenic glycoprotein gp350/220 from EBV (EBVgp350/220 VLP) [99]. The constructed vaccine was examined *in vivo*, and the results indicated that the mice immunized with EBVgp350/220 VLP induced epitope specific immune response and the antibody produced in mice was able to neutralize the EBV *in vitro*. In addition, the antibody type from the immunized mice was predominantly IgG1 subclass, which indicates that the EBVgp350/220 VLP is a potentially promising preventive vaccine and has the tendency to elicit T helper type 2 (Th2) cell's humoral immune response [99]. Another antigen for EBV, EBNA1 was considered to be the primary antigen of the EBV-specific T-cell response [100, 101]. One antigen-antibody complex was constructed by chemically conjugating EBNA1 with aDEC-205 dendritic cells (aDEC-205/EBNA1). EBNA1-specific $CD4^+$ and $CD8^+$ T cells were detected in EBV positive patients. Also, humanized mice injected with aDEC-205/EBNA1 complex successfully developed EBNA1-specific IgM and T-cell responses [102]. These findings suggest that glycoprotein gp350/220 and EBNA1 could be used as the potential target epitopes for the development of EBV vaccines. However, the stability and immunogenicity of EBV

vaccines still need to be proved and then improved. In addition, there is no licensed EBV vaccine available yet.

2.4.2 Hepatitis C virus (HCV)

It is reported that approximately 170 million people have been infected with HCV globally. Normally the early stage of the HCV infection has no obvious symptom, however, it often leads to liver disease after long term infection [103]. Around 60-80 % of the people develop a long-term liver disease. 11% of these patients have the risk to deteriorate to liver cancers [104-106]. In addition, the reinfection of HCV is another major challenge for long-term protection [107]. The transit pathway for HCV is mainly by blood transmission [108]. **Figure 2.5** illustrated the structure of HCV. HCV is a small enveloped, positive-sense single-stranded RNA virus of the family *Flaviviridae* with the size of 55–65 nm in size [109]. It is composed of E1 and E2 glycoproteins, lipid envelope layer, capsid layer (core protein), and viral genome [110].

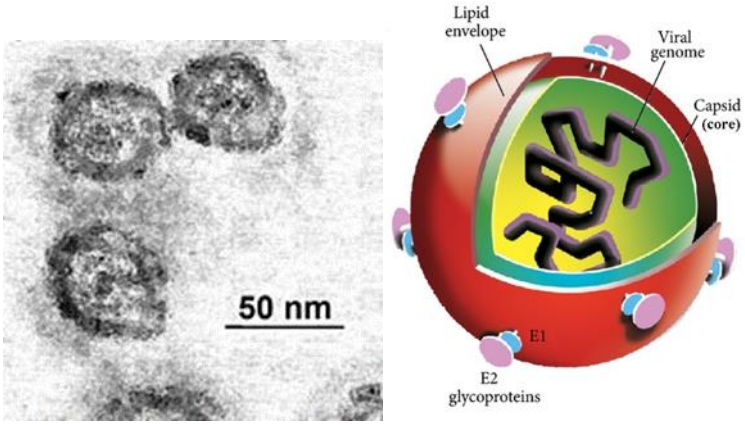

Figure 2.5 TEM image and schematic representations of HCV [111]

For the development of vaccines against HCV, HCV-VLPs are reported to be a promising platform against HCV infection and related diseases. HCV-VLPs are

normally constructed using viral glycoproteins E1 and/or E2 (as shown in **Figure 2.5**). Various animal models including mouse and baboon have been tested for the immunogenicity efficiency of HCV-VLPs and satisfied results were obtained [112-115]. Recently, the protective efficacy of HCV VLPs was evaluated against chimpanzees, the only HCV susceptible animal model. E1- and E2-specific humoral and cell-mediated immune responses were successfully elicited, and protection ability of the vaccinated animals was achieved in the HCV challenge experiment [116]. However, the immune response level and long-term protection ability still need to be improved compared with other commercial vaccines. In addition, VLPs such as Hepatitis B surface antigen (HBsAg) VLP have been developed to present the epitope of hypervariable region 1 (HVR1) of HCV to form HVR1-HBsAg VLP [117]. In the immunization experiment, chimeric HVR1-HBsAg VLP successfully induced HVR1 specific humoral immune response in the mice after vaccination. Also, *in vitro* studies confirmed that after vaccination, it showed effective protection against HCV [117]. In the meantime, HBsAg specific immune response in mice was also detected. This finding potentially explores the development of multivalent hepatitis vaccines. However, long-term protection was not conducted in this research. Another epitope, HCV core, is reported to be potentially employed for the development of HCV vaccines, since this is the most highly conserved region of the translated HCV genome both within, and between, different HCV genotypes [118]. Recently, a peptide derived from HCV core region (C35–44) was investigated in a Phase I in Japan [119]. The results demonstrated that

60 % patients were detected to have peptide specific $CD8^+$ activity and greater than 30% patients had increase in alanine transaminase which proves the improvement of vaccination efficacy. With these findings, HCV core epitope and glycoproteins E1 and E2 can potentially be applied as the target epitopes in the development of vaccines against HCV infection and related diseases. HCV vaccines with effective and long-term immune response should be further explored.

2.5 Chimeric VLP vaccines

In the development of VLP vaccines for the oncoviruses, researchers found that not all viral capsid proteins are suitable to form VLP vaccines. In this case, chimeric VLPs have been developed by adding foreign antigens through genetic modification or chemical coupling to the selected VLP. Chimeric VLPs can present multiple antigens and potentially target to multiple viruses. Several VLPs including HBc, HPV, Porcine Circovirus (PCV) and Murine polyomavirus VP1 (MuPyV) have been employed as the carrier of different antigens to form the chimeric VLP vaccines. Therefore, rational design and production of chimeric VLPs are crucial for future development and applications in clinical practice for cancer treatment. **Table 2.5** lists some recent research in chimeric VLP vaccines development. The VLP carrier, presented antigen, host cell, target virus of disease and purification process have been summarized in the table and challenges of production of these VLP vaccines are briefly provided. Firstly, some chimeric VLP proteins were expressed in inclusion bodies in bacteria expression system [78, 120, 121]. This can increase the complexity in the purification and

assembly process. For example, low recovery yield of chimeric VLP protein is reported [122]. Secondly, to improve the purification efficiency, affinity His-tag was normally fused to the chimeric VLP proteins. However, His-tag need to be removed when preparing the chimeric VLPs as vaccine candidates in clinical trial due to its possibility to impact on the vaccine immunogenicity [123]. Further tag-free purification process must be developed. The molecular complexity of chimeric VLPs and the maintenance of the structure of chimeric VLPs during purification process are the main challenges. Different purification strategies need to be designed regarding different chimeric VLP proteins. Finally, when applying genetic fusion strategy for insertion of foreign epitopes, insertion position needs to be considered according to different VLP carriers. Ideally, the inserted antigens are preferred to be exposed on the surface of chimeric VLPs to maximize their immunogenic potential. A buried antigen could influence the immunogenicity performance [124]. Details of production process of chimeric VLP vaccine and challenges in each step are reviewed in **section 2.7**.

Table 2.5 Recent productions of chimeric VLP-based vaccines.

VLP	Carried antigen	Host cell	Target virus or disease	Purification process	Challenges in production	Year	Ref.
Truncated HBc	NadA (MIR) FHbp (C-terminal)	BL21 ClearColi *E. coli* (bacteria)	*Neisseria meningitidis*	Strep-Tag affinity chromatography	1. Yield of chimeric VLP was around 8mg per litre of cultured cells. 2. Melting point (Tm) of chimeric VLPs reduced due to the insertion of foreign antigen. 3. Foreign antigens had to be inserted in specific position to avoid compromising VLP expression and assembly. 4. Fusion tag was used.	2020	[122]
Truncated HBc	CD8+ cell epitope (HF10 or ROP7) and a B cell epitope (SAG182−102 or SAG1301−320) of T. gondii (MIR)	BL21 *E. coli* (DE3) (bacteria)	*Toxoplasma gondii*	Inclusion body renaturation and Ni_2^+ iminodiacetic acid (IDA) affinity chromatography	1. Target chimeric VLP proteins were expressed in the inclusion bodies in *E. coli*. 2. Fusion tag was used.	2019	[121]
Truncated HBc	B cell epitopes on the VP1 of FMDV and a T cell epitope on non-structural protein 3A （MIR）	BL21 *E. coli* (DE3) (bacteria)	Type A and O foot-and-mouth disease virus	Inclusion body renaturation, Ni SepharoseTM chromatography and in vitro assembly of VLPs	1. Chimeric VLP proteins were expressed in the inclusion bodies in *E. coli.* 2. Affinity tag was used.	2019	[120]
Full length HBc	VP2-P28 (MIR)	BL21 *E. coli* (DE3) (bacteria)	Coxsackievirus A10 (CVA10)	Polyethylene glycol (PEG) precipitation and sucrose gradient	1. Cost of sucrose gradient ultracentrifugation is high.	2019	[125]

				ultracentrifugation	2. Low temperature induction was applied for soluble expression of recombinant chimeric VLP proteins.		
Truncated HBc	SP70 (MIR)	BL21 *E. coli* (DE3) (bacteria)	Enterovirus 71 (EV71)	DEAE-Sepharose fast flow ion exchange chromatography and cesium chloride (CsCl) cushion and gradient centrifugation	N/A	2018	[126]
Truncated HBc	Influenza H3N2 virus HA stalk domain (MIR)	*P. pastoris* KM71H (yeast)	Influenza virus	Polyethylene glycol (PEG) precipitation, Sepharose 4 FF size exclusion chromatography and Fractogel EMD TMAE (M) anion exchange chromatography	1. Aggregation of chimeric VLP proteins in PEG precipitation was found and they used urea to avoid the aggregations. 2. Hetero-tandem core technique was applied to avoid the insoluble expression of chimeric HBc VLP proteins.	2017	[127]
Truncated HBc	EV71-VP1, EV71-VP2, and VZV-gE (MIR)	ER2566 *E. coli* (DE3) (bacteria)	Varicella-zoster virus (VZV) and enterovirus 71 (EV71)	Ammonium sulphate precipitation, TSK-GEL SW3000 gel filtration chromatography and DEAE-5PW anion exchange chromatography	1. Some impurity proteins from host cells remain after purification process.	2017	[128]
Full length HBc	SP55 or SP70 peptides from EV71 (MIR)	BL21 *E. coli* (DE3) (bacteria)	Enterovirus 71 (EV71)	Inclusion body renaturation and Ni-NTA His-Bind affinity chromatography	1. Recombinant proteins were highly expressed as inclusion bodies. 2. Affinity tag was used.	2014	[78]
Truncated HBc	pre-S1 (MIR) HCV core (C-	K802 *E. coli* (bacteria)	Hepatitis B virus (HBV)	Ammonium sulphate precipitation, Sepharose CL-4B	1. The expression level of chimeric proteins was around 5% of the total intracellular protein.	2010	[124]

	terminal)		and hepatitis C virus (HCV)	size exclusion chromatography	2. HCV core epitope was buried within the particles		
Macrobrachium rosenbergii nodavirus (MrNV) VLP	hepatitis B virus "a" determinant (aD) (C-terminal)	Sf9 (insect cell)	Hepatitis B virus	His-Trap HP affinity chromatography	1. The sizes of expressed VLPs are not uniformed and are not the same compared with the one expressed in *E. coli*. 2. Affinity tag was used.	2020	[129]
HPV16 L1 VLP	HPV31 L2	Bac-to-Bac baculovirus expression system	Human papillomavirus (HPV)	Cesium chloride (CsCl) ultracentrifugation	1. Secondary structure of 31RG-1 peptide in the DE loop was different from that in the h4 coil regions of HPV16 VLPs.	2018	[130]
HPV16 L1 VLP	β-amyloid 11–28 epitope (Aβ 11–28)	*Agrobacterium tumefaciens* GV3101 (plant)	Amyloid-beta (Aβ)	HiTrap Capto S ImpAct ion exchange chromatography and Superdex 75 size exclusion chromatography	1. Conformation of chimeric VLP is not clear. 2. Expression level is low.	2018	[131]
Porcine Circovirus Type 2 VLP	Middle portion of Loop CD (MP-Lcd)	BL21 *E. coli* (DE3) (bacteria)	Porcine circovirus (PCV)	HisTrap^{TM} HP chromatography	1. Substitution of MP-Lcd with a foreign peptide caused surface pattern changes around two-fold axes of PCV2 VLPs based on 3D structure simulation. 2. Affinity tag was used.	2018	[132]
Murine polyomavirus VP1 VLP	capsid protein of porcine circovirus 2b (PCV2b)	Baculovirus/ insect cell	porcine circovirus 2 (PCV2)	Affinity chromatography	1. Large aggregation formed in the isolation process. 2. Affinity tag was used.	2017	[133]

2.6 Computational protein modelling for study of novel chimeric VLPs

The design and production of novel chimeric VLP-based vaccines are normally believed to be complicated due to the consumption of time and money [134]. In addition, the structure stability and interaction between the subunits of chimeric VLPs play a crucial rule in the development of successful chimeric VLP-based vaccines [12]. To reduce the time consumption and improve the efficiency in vaccine development process, computational protein modelling was applied to improve the VLP sequence design and understand the process of assembly of chimeric VLPs. As shown in **Figure 2.6**, the process of computational protein modelling includes 1) VLP structure construction using structure determination methods such as X-ray crystallography, cryo electron microscopy (cryoEM) and Nuclear Magnetic Resonance (NMR), 2) novel structure construction model using computational tools such as bioinformatics, homology modelling and fold recognition, 3) study of the interaction between subunits of novel VLP proteins using computational tools such as docking and molecular dynamic (MD) simulation, and 4) comparison of the results obtained from computational protein modelling with the results achieved in experiments. As reported, computational protein modelling has been extensively applied to biological research and drug discovery [135, 136], however, their application in VLP-based vaccines is still in the early stage.

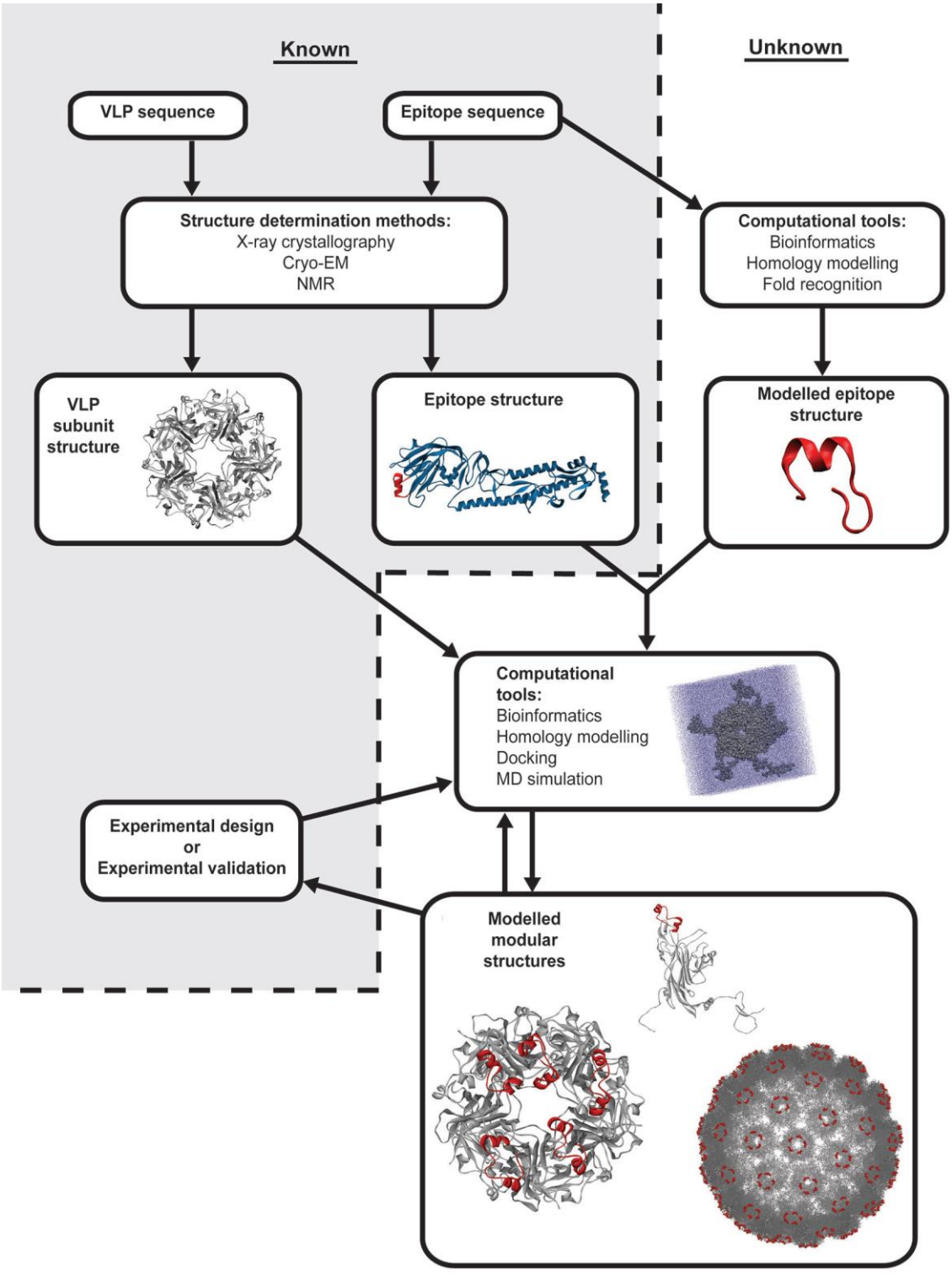

Figure 2.6 Application of computational tools for modular VLP design. [12]

2.6.1 Structure determination of chimeric VLP

To perform the computational protein modelling for the guidance of design and stability study of chimeric VLP, it is believed that the first step is the modelling of structure of

VLP proteins [137]. Several structure determination methods including X-ray crystallography, cryoEM and NMR could be applied to form the structure files of target VLP samples for computational modelling. For example, truncated HBc VLP structure was determined using X-ray crystallography to 3.3Å resolution in 1999 [138]. The structure of full length of HBc VLP was determined using cryoEM to 3.5Å resolution [139]. In addition, other VLPs such as Coxsackievirus A6 (CVA6) VLPs [140] and porcine circovirus type 2 (PCV2) VLPs [141] were determined using cryoEM and HPV VLPs [142] were determined by X-ray crystallography. These determined VLP protein structures can be download from RCSB Protein Data Bank website. **Figure 2.7** illustrates the 3D structure of monomer of HBc and assembled HBc VLP (PDB ID: 3J2V). These VLP structures are the basis for the construction of chimeric VLPs by addition of foreign antigen structures. However, when adding foreign antigens to the determined VLP structures, the chimeric VLP structure could be different from the real structure and got incorrect results in the simulation later.

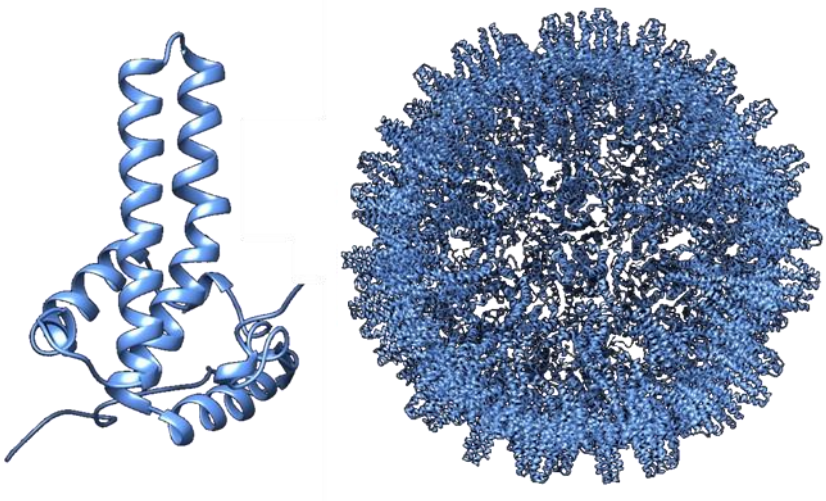

Figure 2.7 3D structure of HBc monomer (left) and assembly (right) (PDB ID: 3J2V).

2.6.2 Molecular dynamic (MD) simulation for the interaction between subunits of chimeric VLPs

After getting the structure file of chimeric VLP protein, further MD simulation is needed to analyse the design of the VLPs [143]. Molecular simulation technique was developed following the rapid evolution of modern computer science [144]. To date, it has become a flexible tool to deliver clear microscopic bioinformation which was applied in the study of protein conformation transition to assist the experimental and theoretical studies [145]. It is reported that important information related to the assembly process of VLPs including nucleus size, average nucleation rate, and average free energy of association could be calculated through simulation process now [143]. With this calculated information, the process of assembly of VLPs as well as the factors that impact on their stability can be understood, which present essential clues to control and manage the self-assembly process of VLPs. Theoretical calculation or numerical simulation is usually related to mathematical models to explore the macroscopic thermodynamic or dynamic parameters for evaluating the self-assembly process. Thermodynamic of VLPs during the assembly process have been investigated using molecular simulation technique. A minimal model was employed to investigate the thermodynamic information of VLPs [146]. The energy change during the assembly process of VLPs was successfully simulated using 'the piece model' consisting of pentagonal and hexagonal pyramids [147]. In addition to the energy, the molecular interactions during the assembly progress were also revealed in the repulsive sites in pyramids. To further investigate the self-assembly process of VLPs, dynamic

parameters were evaluated using MD simulation. However, in the process of the simulation, whole VLP model is thought to be too large. The simulation was then successfully proceeded with a reduced model consisting of simplified trapezoidal capsomere structure of chimeric VLP and this structure was examined by MD simulation [148, 149]. Another similar model was composed of triangle pieces from VLPs was constructed and the kinetic trapping effects in self-assembly processes was successfully calculated [150]. However, it is still tough to reveal and control the self-assembly process of VLPs using computational approach. Several issues such as the computational accuracy, the impact of the actual environment and large calculation due to the massive atom numbers of VLPs are remained [143, 151]. Improvement and development of computer hardware as well as the calculation algorithm and processing capacity are essential to make the application of computational protein modelling possible for the design and understand of VLP-based vaccine in the future. However, with current hardware and calculation algorithm, the interaction and assembly process of the subunits of whole VLPs such as HPV VLPs and HBc VLPs are still hard to complete.

2.7 Production and challenges of chimeric VLP vaccines

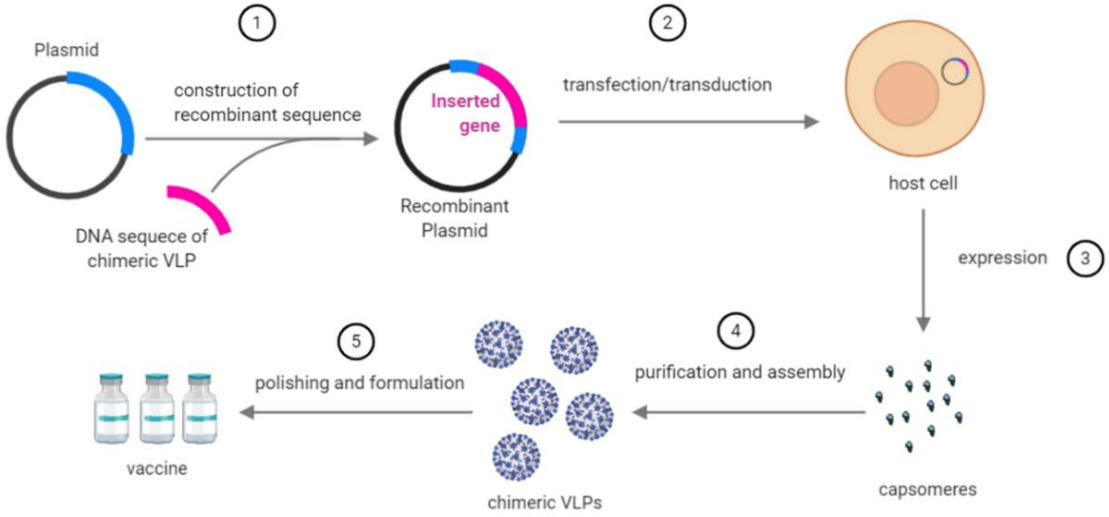

Figure 2.8 Production process of chimeric VLP vaccine

In general, as shown in **Figure 2.8**, a successful production process of chimeric VLP vaccine includes: 1) construction of recombinant VLP sequence with gene engineering techniques, 2) transfection or transduction to the selected host cell, 3) expression in the selected host cells, 4) purification and assembly of expressed capsomeres of chimeric VLP and 5) polishing and formulation of assembled chimeric VLP to form the final vaccine product [12].

Chimeric VLPs are constructed by inserting foreign epitopes to well-studied VLPs that have been developed. **Figure 2.9** illustrates the process of production for different types of chimeric VLPs including non-enveloped and enveloped VLPs. In **Figure 2.9a**, construction of non-enveloped chimeric VLPs by genetic fusion is displayed. Briefly, target antigen sequence was genetically fused with VLP sequence and expressed in the host cells such as mammalian cells, insect cells, yeasts, bacteria. In **Figure 2.9b**, non-enveloped chimeric VLPs were produced via chemical conjugation. Non-enveloped VLPs were expressed and then foreign epitopes were conjugated to the surface of VLPs

using various approaches such as disulphide bond formation, covalent conjugation and chemical cross-linking [152-154]. In **Figure 2.9c**, more complicated enveloped chimeric VLPs were produced via the protein transfer approach. Enveloped VLPs were expressed by host cells, and then mixed with the glycosylphosphatidylinositol (GPI)-anchored proteins. Then, target antigens were added to the lipid bilayer of the VLPs to form the chimeric enveloped VLPs [7]. The licensed HBV and HPV VLP-based vaccines are all non-enveloped vaccines [10], while enveloped VLPs have been applied in study for Influenza vaccines [155, 156]. Compared with traditional egg-based production process of inactivated flu vaccine, production process of chimeric VLP vaccine is safer and with rapid production time. However, even though chimeric VLP vaccines were successfully produced, there are still a lot of challenges in the production of chimeric VLPs such as 1) soluble expression of VLP samples to avoid complicated renaturation process, 2) conformation and structure stability of diversity of chimeric VLPs and 3) immunogenicity of chimeric VLPs after insertion of foreign epitopes.

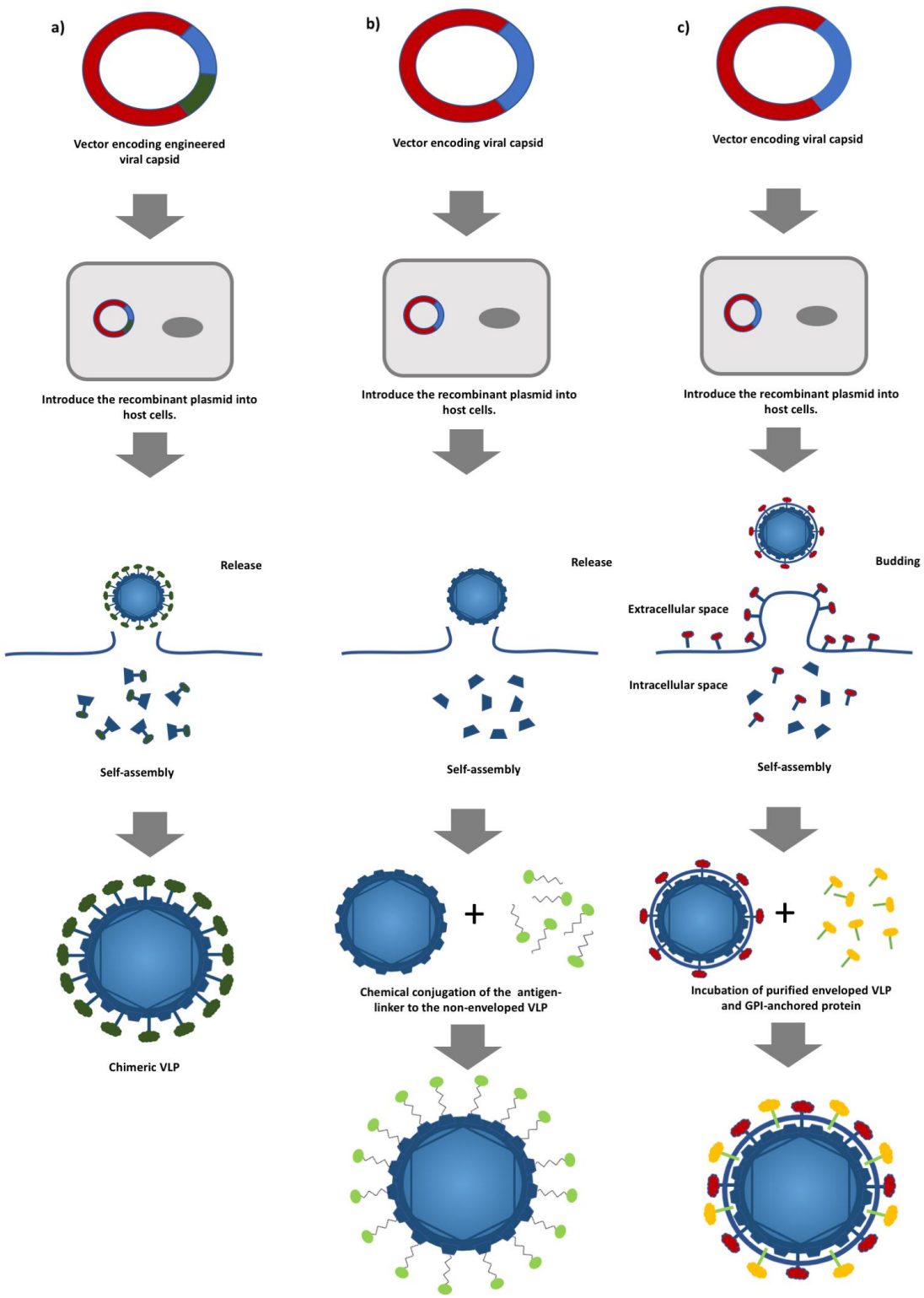

Figure 2.9 Methods to produce different types of chimeric VLPs. [7]

2.7.1 Design and construction of recombinant sequence

The first step to produce chimeric VLP vaccines is to construct the recombinant DNA sequence. The design and construct of chimeric VLP protein are vital to guarantee its

VLP conformation and specific immunogenicity [53]. To maximize the immunogenic potential of chimeric VLP vaccines, target antigens are desired to be presented on the surface of chimeric VLPs. Genetic fusion and chemical conjugation of target antigens are two popular approaches to engineer VLPs for the antigen presentation [157]. To perform the genetic fusion, foreign antigens are fused to the insertion positions or conjugation sites within the selected viral structural protein. These insertion positions or sites need to be able to present the foreign antigens on the surface of VLP. However, genetic fusion can affect the ability of chimeric proteins to assemble into VLPs [158, 159]. In this case, the selection of insertion position in VLP proteins needs extra attention. In addition, the stability of VLPs after insertion of foreign epitopes is another concern in the design and construction [160]. To avoid these issues, foreign antigens can be conjugated to the selected VLP candidate through various conjugation techniques. Amino groups that are exposed on the surface can be suitable targets for the conjugation, or VLPs can be engineered to obtain some functional groups on the surface by post-production modifications.

2.7.2 Transfection and transduction

Before the host cells could express the target VLP proteins, plasmid containing antigen and VLP coding sequence needs to be transferred to the host cells. For different host cells, different approaches have been developed. Heat shock and electroporation are two popular methods to transduce the plasmid to bacteria host cells [161]. Virus infection method are often used to deliver the plasmid to insect and mammalian cells [162]. Successful transfection and transduction of recombinant chimeric VLP plasmid

to the host cell is essential for the whole production. Standard procedures for heat shock and electroporation have been made and optimized for decades.

2.7.3 Expression of chimeric VLPs

Table 2.6 Statistics percentage for different expression system employed in VLP production [163]

Expression system	Percentage (%)
Bacteria	28
Yeast	20
Insect cells	28
Mammalian cells	15
Plant	9

Table 2.7 Comparison of different host cells for expression of chimeric VLPs [164].

Host cells	Advantages	Disadvantages
E. coli	• Ease of expression • Ability to scale-up • Low production cost	• Does not allow for glycosylation. • Endotoxins • Inclusion body
Yeast	• Ease of expression • Ability to scale-up • Low production cost	• Non-appropriate protein glycosylation (i.e., high mannose glycoprotein modification). • Risk of incorrect folding & assembly.
Baculovirus/insect cell (B/IC)	• Can produce large amounts of correctly folded VLP in high density cell culture conditions • Ability to scale-up • The risk of culturing opportunistic pathogens is minimized compared to mammalian cell culture • Host-derived insect cell/baculovirus components may act as vaccine adjuvants, help trigger a more effective immune response	• Limited to high mannose glycoprotein modification. • Baculovirus contaminants may be difficult to remove • Host-derived insect cell/baculovirus components may also mask the immune response against the desired epitope
Mammalian cells	• Producer cells more closely related to the natural host • Appropriate post-translational modification (PTMs) and authentic assembly of VLPs	• Higher production cost • Lower productivities
Plants	• Ease of expression • Ability to scale-up • No human-derived virus contamination	• Cannot undergo PTMs and VLP assembly • Low expression levels • Stability: antigen degradation

VLP-based vaccines can be expressed both by prokaryotic and eukaryotic systems including bacteria, yeasts, insect cells, mammalian cells and plants. The percentage of the application of different expression system is revealed in **Table 2.6** [163]. Bacteria, yeasts and insect cells are three major host cells used for the production of chimeric VLPs. The commercial HPV vaccines based on L1 capsid protein are produced by eukaryotic systems such as yeasts (Gardasil) and insect cells (Cervarix) [165]. The commercial Hepatitis B vaccines, Recombivax and Engerix-B, are also produced in yeast, and are composed of HBsAg [166]. Different expression systems possess their own advantages and disadvantages. For the expression for VLPs, the structural complexity and immunogenicity of the desired VLPs are key determinants for the choice of expression system for VLP production. **Table 2.7** demonstrates the advantages and disadvantages of different expression host cells, and the details are in following sections.

2.7.3.1 Bacteria

Bacteria is regarded as a popular prokaryotic system to express non-enveloped chimeric VLPs composed of one or two capsid proteins. The advantages of bacteria expression system are 1) high yield of target proteins, 2) ability to be scaled up and 3) low cost. For example, *Lactobacillus casei* has been successfully applied in the production of HPV Type 16 L1 VLPs. The presentation of the antigen in the produced VLP protein was detected using immunofluorescence [167]. *Escherichia coli* (*E. coli*) was another popular type of the bacteria used for the expression of VLPs. It has been successfully used in the production of several VLP proteins including recombinant norovirus VLP

[168], recombinant HBc VLP [78, 125, 128], and recombinant HPV VLP [169, 170]. However, lack of post-translational modification (PTM) and the endotoxins from host cells potentially influence the immunogenicity performance of produced VLP vaccines [171]. In addition, some chimeric VLP proteins are reported to be expressed in the inclusion bodies (inactive proteins aggregates) by *E. coli* expression system [78, 172, 173]. It is more complicated for the downstream processing to deal with inclusion bodies as VLP proteins need to be refolded to the correct conformation and assemble to VLP structure *in vitro*. In this case, efforts are made to express the VLP proteins in the soluble form. Some fermentation conditions have been investigated to improve the expression of soluble VLPs. For example, low temperature fermentation has been employed in the production of infectious hypodermal and hematopoietic necrosis virus (IHHNV) [174] and potyvirus, Potato virus Y (PVY) [175]. In addition, the concentration of inducer [176], composition of culture medium [177] and fusion of tag like small ubiquitin-like modifier (SUMO) [178, 179] have been explored to improve the expression of soluble VLPs. Bacteria expression system is recognized as a promising expression system for the production chimeric VLP vaccines. However, optimization of the fermentation conditions to express the chimeric VLP proteins as soluble VLP proteins are essential.

2.7.3.2 Yeast

As described above, two of the commercial VLP vaccines are produced using eukaryotic system such as yeast. Similar to bacteria expression system, yeast expression system obtains the ability to scale up and can produce VLP protein in high yield. In

addition, yeast can also perform post-translational modifications which makes it capable to produce more complicated molecules. For example, *P. pastoris* strain was reported to produce the Chikungunya VLPs and the produced VLP vaccine had good murine immunisation performance [180]. RNA bacteriophage VLPs were also reported to be successfully expressed in *Pichia pastoris*. However, the expression level was only 30% compared with the bacteriophage VLPs expressed in bacteria expression system [181]. Besides, when applying yeast expression system for enveloped VLP production, Morikawa et al. (2007) reported that yeast expression system missed some necessary host factors such as endosomal sorting molecules TSG101, Nedd4, AIP-1/
ALIX, and AP-3 to support the formation of enveloped VLPs [182]. Moreover, VLP proteins expressed by yeast expression system do not always present as assembled VLP structure in the cultivation. Lünsdorf et al. (2011) indicated that HBsAg expressed by *Pichia pastoris* cannot form assembled VLP *in vivo*, and the *in vitro* assembly was conducted during the purification downstream process [183]. It brings extra steps in downstream process.

2.7.3.3 Baculovirus/insect cell (B/IC)

B/IC expression system are composed of two steps, infection step and production step. The designed plasmid is transduced to the Baculovirus in the first step and then selected insect cells are infected by Baculovirus for the production of chimeric VLPs. The advantages of B/IC expression system are 1) rapid growth rate in animal product-free media, 2) ability for large-scale production, and 3) good ability of post-translationally modification of chimeric VLP proteins [184]. The insect cell expression system can

produce more complicated or enveloped chimeric VLP compared with bacteria and yeast expression system due to its ability of better post-translationally modification [171]. Sf9 (*Spodoptera frugiperda*) and High Five cells (*Trichoplusia ni*) are two popular insect cells that are applied in the production of VLP proteins. Chikungunya, HIV or porcine parvovirus VLPs have been successfully expressed in B/IC expression system. The major challenge for B/IC expression system is the baculoviruses are produced with the production of VLP proteins. It increases the cost and hardness to separate baculovirus from VLP mixture. Replacing baculovirus with other transfection reagent can be one option to overcome this challenge. Shen et al. (2014) reported that they successfully used polyethyleneimine (PEI) to replace the baculovirus as the transfection reagent to transfer a plasmid carrying the homologous region 5 enhancer (hr5) and the immediate early 1 (ie1) promoter from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV) to Sf9 cells for the production of tumour necrosis factor receptor (ectodomain) fused to an Fc domain (TNFR-Fc) [185]. This can potentially solve the issue of baculovirus impurities in the purification.

2.7.3.4 Mammalian cells

It has been demonstrated that the production level of VLP proteins of mammalian cell expression system is lower compared with other expression systems. However, mammalian cells have more advantages to produce complex and accurate post-translational modification of VLP proteins [186]. In this case, researchers employed mammalian cell expression system to produce complicated enveloped VLPs with multiple capsid proteins. Chinese Hamster Ovary (CHO) cell line [187] and Human

Embryonic Kidney 293 (HEK293) cell lines [188] are two popular mammalian cell expression systems. CHO cell line, as it is not human derived cell line, has a lower risk of contamination by human virus compared with HEK293 cell line [189]. Li et al. (2010) successfully produced Hantaan VLP (HTN-VLPs) using CHO cells and obtained specific immune response in mice [190]. The HEK293 cell line are reported to successfully produce VLPs against rabies [191], HIV [192], and influenza [193]. However, the growth rate and expression level of mammalian cells are lower compared with other expression systems such as bacteria and yeast, which makes it not suitable for the production in large-scale.

2.7.3.5 Plants

Only 9 % of the VLPs are produced in plant system. *Agrobacterium tumefaciens* is one of bacteria used for infection and transformation of the plant cells [194]. These bacteria are used to introduce the designed gene of VLP protein to the host genome of plant cells. For example, HPV type 16 or influenza are reported to be produced using plant expression system. Several VLP proteins have been expressed using plants, such as HPV VLPs and influenza VLPs [195, 196]. *Nicotiana tabacum* and *Arabidopsis thaliana* [197] are two popular plant expression systems. However, due to the disadvantages including failure of VLP assembly after expression, low expression levels and antigen degradation, plant expression system is not the first option to produce new chimeric VLP [164].

2.7.4 Purification and characterization of chimeric VLPs

After expressing target chimeric VLPs by suitable host cell, chimeric VLP samples

need to be purified for the immunogenicity application. Downstream purification process is essential for the quality of chimeric VLP vaccines as impure samples can cause severe side effects and safety concerns. Purified chimeric VLP vaccines need to meet the requirements including 1) absence of contaminant proteins and DNA (host or viral), 2) absence of incorrectly assembled macrostructures and 3) endotoxin levels below those specified by regulatory agencies (Food and Drug Administration (FDA) and European Medicines Agency (EMEA). However, in the process of purification, similar protein features of impure host cell proteins to target VLP proteins such as size and molecular weight make the process challenging. Purification approaches are designed according to the nature of chimeric VLP proteins such as surface charge, thermostability and tolerance of ion strength and unique characteristics that strongly impact the design of the downstream strategy [198-201].

The stability and the risk of structure mutation of chimeric VLPs during the purification process also need to be taken into consideration [163, 164, 202]. In addition, chimeric VLP proteins produced by different expression systems would contain different impurities and the strategies for the purification vary accordingly. For small scale purification approach, researchers applied ultracentrifugation by sucrose or cesium chloride gradients to obtain small amount of VLP proteins including HBc derived VLPs [126, 203], dengue virus (DENV) VLP [204] and norovirus VLP [205]. Even though the purity of VLP proteins after ultracentrifugation is high, the yield is low compared with other purification process. In addition, the scale-up of ultracentrifugation is challenging. Affinity chromatography attracts the attention of researchers because of

its high specificity. To perform affinity chromatography, an affinity tag needs to be fused to the VLP sequence [206, 207]. However, reports indicate that these fusion tags can potentially affect virion maturation and protein expression, indirectly [208, 209]. The purification process can vary according to the design of different chimeric VLPs as the impact of the insertion of foreign antigens to VLP carrier is different. The main challenge for the purification of chimeric VLP is to maintain its stable VLP structure and avoid the aggregation and disassembly as chimeric VLPs are macromolecules and their structure is essential for their immunogenicity. Different approaches need to be tested to find the optimal purification strategy for new chimeric VLP vaccines. To improve the yield and maintain the purity of VLP proteins, techniques including salting-out, tangential flow filtration, gel permeation chromatography, liquid chromatography, ion exchange chromatography, affinity chromatography, size-exclusion chromatography and disposable membrane technology were developed for the purification of chimeric VLP proteins [210-215].

2.7.5 Polishing and formulation

The final step to produce chimeric VLP vaccines is polishing and formulation. Before injecting chimeric VLP proteins, polishing usually includes 1) removal of final impurities using size-exclusion chromatography, 2) addition of active compounds and 3) exchanging buffer to the final formulation buffer [216]. In the application of vaccines, the addition of adjuvant is regarded as a popular approach to improve the immunogenicity of vaccine candidates [217]. However, one commonly used adjuvant, aluminium adjuvant was reported to have the risk to damage the cells due to the

neurotoxicity [218]. How to safely apply different adjuvants in the formulation of vaccines could be a complicated process. Researchers have found that VLPs obtain the self-adjuvant property as it was assembled by the viral capsid proteins and therefore can induce strong immune response. For example, VLPs with the pathogen associated molecular pattern (PAMP) of viruses can be recognized by Toll like receptors on the surface of cells to induce strong immune response [219, 220]. This self-adjuvant property of chimeric VLPs enables it to be formulated as vaccines without additional adjuvant.

2.8 Characterisation methods for VLPs

During a desired purification processing of VLP-based vaccines, impurities were removed to a level meeting the regulation while maintaining target VLP proteins. However, the structures of VLPs are often important for their biological activities that are vulnerable to environmental stresses in downstream processing. Denaturation or disassembly of VLPs are frequently observed [221, 222]. Such changes of the structure of VLPs not only makes the purification process inefficient but also raise the concerns for their further immunogenicity problems. To avoid denaturation and disassembly of VLPs during downstream processing, characterization techniques have been developed to monitor and evaluate the condition of VLPs. **Table 2.8** listed some common techniques for characterization of VLPs and their functions.

Table 2.8 Common characterization techniques for VLPs.

No.	Technique	Function
1	High performance size exclusion chromatography (HPSEC)- multi-angle laser light scattering (MALLS)	Separate proteins and measure the molecular weight and size
2	Dynamic Light Scattering (DLS)	Measure particle size
3	Circular dichroism (CD)	Determine secondary structure
4	Intrinsic fluorescence (IF)	Determine tertiary structure
5	Differential scanning calorimetry (DSC)	Measure thermal stability
6	Transmission electron microscopy (TEM)	Visualize the structure
7	Differential scanning fluorescence (DSF)	Measure thermal stability
8	Mass spectrometry (MS)	Identify and quantify protein molecules
9	Nuclear magnetic resonance (NMR)	Determine the overall structure
10	Isothermal titration calorimetry (ITC)	Provide a complete thermodynamic profile

2.8.1 High performance size exclusion chromatography (HPSEC)-multi-angle laser light scattering (MALLS)

HPSEC is a non-destructive approach to separate proteins based on their molecular weight. It has been applied in the study of various protein-based biomaterials such as antibody and vaccines. For analysis of VLPs, the major challenge is due to the pore size and resolution of HPSEC resins. VLPs are normally in large particle size and is difficult to discriminate between structural diversities of particles [223]. However, this issue was solved with the development of HPSEC matrix. Several matrixes with large pore size

(more than 100nm) are now commercially available such as TSKgel G5000PWxl and TSKgel G4000PWxl [224]. HPSEC technique has now been applied in the separation of several protein-based molecules such as inactivated foot and mouth disease virus (FMDV) vaccine [225]. The intact FMDV, also called as 146S, can be disassembled into smaller 12S particle leading to lower immunogenicity issue [226]. Therefore, the detection of 146S during the production of FMDV vaccine is essential. HPSEC showed significant convenience and short time consumption in the detection compared with other methods such as conventional ultracentrifugation and ELISA [227]. Similar application of HPSEC can be applied to the production of VLPs. By developing appropriate procedure, different status of VLPs such as aggregation, assembly and disassembly can be quantitatively measured rapidly [228]. Coupling HPSEC with a MALLS detector further expands the application of HPSEC. MALLS detectors determine the weight-average molar mass, average size by detecting the scattered light at multiple angles and extrapolating to 0° [229]. Therefore, HPSEC-MALLS can detect VLPs and aggregates based on the molecular weight and particle size.

2.8.2 Dynamic Light Scattering (DLS)

DLS, also known as photon correlation spectroscopy or quasi-elastic light scattering, is able to determine the virus-like particle size quickly and accurately. DLS has emerged as the simplest and most executable table-top detection method to perform, because it only needs an ordinary lab environment. DLS has developed into popular tools within pharmacy community, according to techniques that were exclusively available to colloid chemists. The compact, integrated and affordable instruments offer user-

friendly digital interfaces, which is along with possibility for the comprehensive data analysis. Furthermore, these techniques are non-invasive, and require minimal sample preparation without pre-experimental calibration. Creative Biostructure has equipped the modern DLS instruments which can not only guide our experienced scientists on the quality of the generated data with possibility for time-dependent measurements but also export the data traces as files compatible with various plotting software to provide you the best research figures.

DLS has a lot of advantages over traditional microscopy such as TEM and AFM because DLS offers the hydrodynamic size of virus-like particles in solution. For example, TEM images often do not substantiate well with the data obtained from DLS. DLS is capable of measuring bigger number of particles (in millions) compared to TEM (few hundreds). Thus, DLS provides more robust data on size distribution and PDI. As a result, DLS is a promising method to measure the size of VLPs and has the potential to be used to process development with upstream and downstream samples.

2.8.3 Circular dichroism (CD)

CD is a method based on the differential absorption of left circularly polarized (LCP) and right circularly polarized (RCP) light by optically active chiral molecules [230]. The difference between the absorbance of LCP and RCP light are recorded and plotted against wavelength. CD spectroscopy has a wide range of applications in many different fields, particularly for evaluating the structure of biomolecules like proteins and polypeptides: CD effects in the far UV ((below 250 nm) range, caused by the protein's amide backbone, provide information about the secondary structure of a protein, due to

characteristic CD bands with positive and/or negative ellipticities at defined wavelengths for each secondary structure element. CD effects in the near UV range (~ 320-250 nm) are due to the presence of aromatic side chains or/and disulphide bridges and reflects the protein tertiary structure. Its spectral profile depends on the type and number of aromatic amino acids and their environment (e.g., degree of hydrogen bonding), therefore it differs substantially between individual proteins, and thus can be considered as a fingerprint region of a protein.

2.8.4 Intrinsic fluorescence (IF)

Tyrosine (Tyr) and Tryptophan (Trp) residues in proteins have unique intrinsic fluorescence. By measuring the intrinsic fluorescence, the tertiary structure of proteins can be revealed [231]. It is reported that differences in intrinsic fluorescence were associated with the transfer of tryptophan residues from a largely aqueous to a largely protein environment [232]. Intrinsic fluorescence has also been applied in the study of VLP structures including Norwalk virus [233] and hepatitis B virus [234]. The advantage of intrinsic fluorescence is that real time data can be obtained for assembly and maturation without chemically modification. However, by only measuring IF, it is hard to differentiate between assembly and maturation of VLPs. More techniques such as DLS, Western blot and transmission electron microscopy (TEM) are needed. In addition, IF is less sensitive to interference by small air bubbles or aggregates that can make sensitive for MALLS analysis [231].

2.8.5 Differential scanning calorimetry (DSC)

DSC is the only technique that can measure the thermodynamics of intra- and inter-

molecular interactions of proteins directly [235]. Therefore, DSC has been employed in the investigation of thermal stability of protein samples. DSC enables to measure the temperature of protein unfolding (Tm), the molar enthalpy change (ΔH), the molar entropy change (ΔS), and the change in the molar heat capacity (ΔCp) accompanying a protein transition. Any change in the conformation of VLP proteins would be revealed by the change of position, sharpness, and shape of transitions in DSC scans [213].

Thermal stability of VLPs can be associated with the design of the purification process. For example, Li et al. developed a purification process of hepatitis B core antigen (HBc) VLP by heated VLP samples at 60°C for 30 min after they found that the Tm value of HBc VLP is around 96.25°C [236, 237]. Around 85.8% particle recovery and 74.7% purity were achieved.

2.8.6 Transmission electron microscopy (TEM)

VLPs, EM has been used as a popular technique to evaluate size, polydispersity, purity and even nanoparticle composition of proteins [238]. Transmission electron microscopy (TEM), scanning electron microscopy (SEM) and cryogenic electron microscopy (cryo-TEM) are three major types for EM. Among them, TEM is reported to be the best technique for the characterization of VLP structure [239]. VLP samples are negatively stained normally using uranyl acetate [240]. Differential electron scattering between the biological material and the surrounding staining layer enables the visualization of the specimen. TEM gives a direct detection of the structure of VLP proteins directly. Aggregation, assembly and disassembly of VLPs can be visually identified. However, sample preparation such as the concentration of VLP samples and

buffer condition as well as the selection of electron microscope can influence the quality of final TEM image.

2.8.7 Other characterization techniques

In addition to the techniques discussed above, Differential scanning fluorescence (DSF), Mass spectrometry (MS), Nuclear magnetic resonance (NMR), Isothermal titration calorimetry (ITC) have contributed to our understanding of the structure of VLPs. Besides various physicochemical techniques, immunochemical methods including ELISA, biosensor analysis and non-labelled immunoassays are also important for characterization of VLPs. These methods are valuable since the integrity of structures is often important for *in vivo* bio-activities [241].

2.9 Summary

Immunotherapy is a promising technology to treat cancers caused by viruses in the modern society. Cancer vaccines are regarded as an approach for cancer treatment with lower side effects compared with traditional cancer treatments. In the development of preventive cancer vaccines, oncoviruses that are related to various cancers globally have been targeted to produce cancer vaccines. Different types of vaccines including traditional and advanced vaccine candidates have been developed. Among these candidates, VLPs, is reported to be one of the most effective and safest candidates for the development of cancer vaccine platform. To apply VLP platform to the treatment of different oncoviruses, recombinant technique has been employed to construct chimeric VLP vaccines by fusing foreign antigens to the selected VLP carriers. In conclusion, the challenges in the development of effective chimeric VLP-based

vaccines for the treatment of oncoviruses leading to cancers includes: 1) design of the sequence of chimeric VLP to expose the antigen on the surface, 2) efficient expression and purification of chimeric VLP proteins using suitable expression system and purification approach, 3) acknowledgment of the factors and mechanisms that influence on the stability and assembly of chimeric VLP proteins and 4) strong antigen specific and long-term *in vivo* immunogenicity.

References

[1] M. Zapatka, I. Borozan, D.S. Brewer, M. Iskar, A. Grundhoff, M. Alawi, N. Desai, H. Sültmann, H. Moch, M. Alawi, C.S. Cooper, R. Eils, V. Ferretti, P. Lichter, I. Borozan, D.S. Brewer, C.S. Cooper, N. Desai, R. Eils, V. Ferretti, A. Grundhoff, M. Iskar, K. Kleinheinz, P. Lichter, H. Nakagawa, A.I. Ojesina, C.S. Pedamallu, M. Schlesner, X. Su, M. Zapatka, P. Pathogens, P. Consortium, The landscape of viral associations in human cancers, Nat. Genet. 52(3) (2020) 320-330.

[2] Oncovirus-Wikipedia. https://en.wikipedia.org/wiki/Oncovirus. (Accessed April 5 2020).

[3] S. Kruger, M. Ilmer, S. Kobold, B.L. Cadilha, S. Endres, S. Ormanns, G. Schuebbe, B.W. Renz, J.G. D'Haese, H. Schloesser, V. Heinemann, M. Subklewe, S. Boeck, J. Werner, M. von Bergwelt-Baildon, Advances in cancer immunotherapy 2019 – latest trends, J. Exp. Clin. Cancer Res. 38(1) (2019) 268.

[4] National Cancer Institute (NIH). https://www.cancer.gov/about-cancer/treatment/types/immunotherapy. (Accessed March 28 2020).

[5] P.-L. Lollini, F. Cavallo, P. Nanni, G. Forni, Vaccines for tumour prevention, Nature Reviews Cancer 6(3) (2006) 204-216.

[6] L.A. Emens, Cancer vaccines: on the threshold of success, Expert Opin Emerg Drugs 13(2) (2008) 295-308.

[7] H.K. Ong, W.S. Tan, K.L. Ho, Virus like particles as a platform for cancer vaccine development, PeerJ 5 (2017) 4053-4053.

[8] J. Cuzick, Gardasil 9 joins the fight against cervix cancer, Expert Review of Vaccines 14(8) (2015) 1047-1049.

[9] J.N. Graff, E.D. Chamberlain, Sipuleucel-T in the treatment of prostate cancer: an evidence-based review of its place in therapy, Core Evid 10 (2015) 1-10.

[10] N. Kushnir, S.J. Streatfield, V. Yusibov, Virus-like particles as a highly efficient vaccine platform: Diversity of targets and production systems and advances in clinical development, Vaccine 31(1) (2012) 58-83.

[11] B. Ma, R. Roden, T.C. Wu, Current Status of Human Papillomavirus Vaccines, Journal of the Formosan Medical Association 109(7) (2010) 481-483.

[12] N.K.C. Linda H.L. Lua, Frank Sainsbury, Yap P. Chuan,Nani Wibowo, Anton P.J. Middelberg, Bioengineering Virus-Like Particles as Vaccines, Biotechnology and Bioengineering 111 (2014) 425-440.

[13] D. Baxter, Active and passive immunity, vaccine types, excipients and licensing, Occup. Med. 57(8) (2007) 552-556.

[14] D. Baxter, Active and passive immunity, vaccine types, excipients and licensing, Occup. Med. (Lond.) 57(8) (2007) 552-6.

[15] A.S. Clem, Fundamentals of vaccine immunology, J. Glob. Infect. Dis. 3(1) (2011) 73-78.

[16] Centers for Disease Control and Prevention (CDC). https://www.cdc.gov/vaccines/vac-gen/immunity-types.htm. (Accessed April 20 2020).

[17] P. National Research Council Division of Health, P. Disease, Vaccine Supply and Innovation, National Academies Press (US)

Copyright (c) National Academy of Sciences., Washington (DC), 1985.

[18] The History of Vaccines. https://www.historyofvaccines.org/content/articles/different-

types-vaccines. (Accessed April 26 2020).
[19] P.D. Minor, Live attenuated vaccines: Historical successes and current challenges, Virology 479-480 (2015) 379-92.
[20] M.D. Wareing, G.A. Tannock, Live attenuated vaccines against influenza; an historical review, Vaccine 19(25-26) (2001) 3320-30.
[21] S. Plotkin, W. Orenstein, P. Offit, Vaccines 6th Edition, 2012.
[22] World Health Organization.
[23] B. Sanders, M. Koldijk, H. Schuitemaker, Inactivated Viral Vaccines, Vaccine Analysis: Strategies, Principles, and Control (2014) 45-80.
[24] Vaccines.gov.
[25] T. Schlake, A. Thess, M. Fotin-Mleczek, K.-J. Kallen, Developing mRNA-vaccine technologies, RNA Biol. 9(11) (2012) 1319-1330.
[26] N. Pardi, M.J. Hogan, F.W. Porter, D. Weissman, mRNA vaccines — a new era in vaccinology, Nature Reviews Drug Discovery 17(4) (2018) 261-279.
[27] K. Karikó, H. Muramatsu, F.A. Welsh, J. Ludwig, H. Kato, S. Akira, D. Weissman, Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability, Mol. Ther. 16(11) (2008) 1833-40.
[28] K. Karikó, H. Muramatsu, J. Ludwig, D. Weissman, Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA, Nucleic Acids Res 39(21) (2011) e142.
[29] S. Guan, J. Rosenecker, Nanotechnologies in delivery of mRNA therapeutics using nonviral vector-based delivery systems, Gene Ther. 24(3) (2017) 133-143.
[30] K.J. Kauffman, M.J. Webber, D.G. Anderson, Materials for non-viral intracellular delivery of messenger RNA therapeutics, J. Control. Release 240 (2016) 227-234.
[31] L.R. Baden, H.M. El Sahly, B. Essink, K. Kotloff, S. Frey, R. Novak, D. Diemert, S.A. Spector, N. Rouphael, C.B. Creech, J. McGettigan, S. Khetan, N. Segall, J. Solis, A. Brosz, C. Fierro, H. Schwartz, K. Neuzil, L. Corey, P. Gilbert, H. Janes, D. Follmann, M. Marovich, J. Mascola, L. Polakowski, J. Ledgerwood, B.S. Graham, H. Bennett, R. Pajon, C. Knightly, B. Leav, W. Deng, H. Zhou, S. Han, M. Ivarsson, J. Miller, T. Zaks, Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine, New England Journal of Medicine 384(5) (2020) 403-416.
[32] F.P. Polack, S.J. Thomas, N. Kitchin, J. Absalon, A. Gurtman, S. Lockhart, J.L. Perez, G. Pérez Marc, E.D. Moreira, C. Zerbini, R. Bailey, K.A. Swanson, S. Roychoudhury, K. Koury, P. Li, W.V. Kalina, D. Cooper, R.W. Frenck, L.L. Hammitt, Ö. Türeci, H. Nell, A. Schaefer, S. Ünal, D.B. Tresnan, S. Mather, P.R. Dormitzer, U. Şahin, K.U. Jansen, W.C. Gruber, Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine, New England Journal of Medicine 383(27) (2020) 2603-2615.
[33] P.M. Moyle, I. Toth, Modern subunit vaccines: development, components, and research opportunities, ChemMedChem 8(3) (2013) 360-76.
[34] L. Nemchinov, T. Liang, M. Rifaat, H. Mazyad, A. Hadidi, J. Keith, Development of a plant-derived subunit vaccine candidate against hepatitis C virus, Archives of virology 145(12) (2000) 2557-2573.
[35] D. Perez-Filgueira, P. Resino-Talavan, C. Cubillos, I. Angulo, M. Barderas, J. Barcena, J.

Escribano, Development of a low-cost, insect larvae-derived recombinant subunit vaccine against RHDV, Virology 364(2) (2007) 422-430.
[36] M. Wang, S. Jiang, Y. Wang, Recent advances in the production of recombinant subunit vaccines in Pichia pastoris, Bioengineered 7(3) (2016) 155-165.
[37] M. Nair, Protein conjugate polysaccharide vaccines: challenges in development and global implementation, Indian journal of community medicine : official publication of Indian Association of Preventive & Social Medicine 37(2) (2012) 79-82.
[38] J.I.C. Alvarez, E.C. Valdes, A.I.R. Casares, C.V. Olmo, K. Dalsgaard, Recombinant subunit vaccine against porcine parvovirus, Google Patents, 1996.
[39] S.L. Stephen, L. Beales, H. Peyret, A. Roe, N.J. Stonehouse, D.J. Rowlands, Recombinant Expression of Tandem-HBc Virus-Like Particles (VLPs), Methods Mol Biol 1776 (2018) 97-123.
[40] D.B. Young, 6 - DISEASE STATES AND VACCINES: SELECTED CASES: PART H. Tuberculosis, in: B.R. Bloom, P.-H. Lambert (Eds.), The Vaccine Book, Academic Press, San Diego, 2003, pp. 279-289.
[41] A.N. Tsoras, J.A. Champion, Protein and Peptide Biomaterials for Engineered Subunit Vaccines and Immunotherapeutic Applications, Annual Review of Chemical and Biomolecular Engineering 10(1) (2019) 337-359.
[42] M.R. Batzloff, K.S. Sriprakash, M.F. Good, Vaccine development for group A streptococcus infections and associated disease, Curr. Drug Targets 5(1) (2004) 57-69.
[43] S. Pruksakorn, B. Currie, E. Brandt, D. Martin, A. Galbraith, C. Phornphutkul, S. Hunsakunachai, A. Manmontri, M.F. Good, Towards a vaccine for rheumatic fever: identification of a conserved target epitope on M protein of group A streptococci, Lancet 344(8923) (1994) 639-42.
[44] P. Richmond, L. Hatchuel, M. Dong, B. Ma, B. Hu, I. Smolenov, P. Li, P. Liang, H.H. Han, J. Liang, R. Clemens, Safety and immunogenicity of S-Trimer (SCB-2019), a protein subunit vaccine candidate for COVID-19 in healthy adults: a phase 1, randomised, double-blind, placebo-controlled trial, Lancet 397(10275) (2021) 682-694.
[45] M. Skwarczynski, I. Toth, Peptide-Based Subunit Nanovaccines, Curr. Drug Del. 8 (2011) 282-9.
[46] M. Skwarczynski, I. Toth, Recent advances in peptide-based subunit nanovaccines, Nanomedicine (London, England) 9 (2014) 2657-69.
[47] P. Simerska, P.M. Moyle, I. Toth, Modern lipid-, carbohydrate-, and peptide-based delivery systems for peptide, vaccine, and gene products, Med. Res. Rev. 31(4) (2011) 520-47.
[48] C. Adessi, C. Soto, Converting a peptide into a drug: strategies to improve stability and bioavailability, Curr. Med. Chem. 9(9) (2002) 963-78.
[49] D.K. Malik, S. Baboota, A. Ahuja, S. Hasan, J. Ali, Recent advances in protein and peptide drug delivery systems, Curr Drug Deliv 4(2) (2007) 141-51.
[50] B.D. Gessner, Q. Jiang, C.H. Van Werkhoven, H.L. Sings, C. Webber, D. Scott, W.C. Gruber, D.E. Grobbee, M.J.M. Bonten, L. Jodar, A post-hoc analysis of serotype-specific vaccine efficacy of 13-valent pneumococcal conjugate vaccine against clinical community acquired pneumonia from a randomized clinical trial in the Netherlands, Vaccine 37(30) (2019) 4147-4154.

[51] L.A. Mandell, 297 - Streptococcus Pneumoniae Infections, in: L. Goldman, A.I. Schafer (Eds.), Goldman's Cecil Medicine (Twenty Fourth Edition), W.B. Saunders, Philadelphia, 2012, pp. 1820-1823.
[52] C.C. Daniels, P.D. Rogers, C.M. Shelton, A Review of Pneumococcal Vaccines: Current Polysaccharide Vaccine Recommendations and Future Protein Antigens, J Pediatr Pharmacol Ther 21(1) (2016) 27-35.
[53] J. Chroboczek, I. Szurgot, E. Szolajska, Virus-like particles as vaccine, Acta Biochim Pol 61(3) (2014) 531-9.
[54] R. Noad, P. Roy, Virus-like particles as immunogens, Trends Microbiol. 11(9) (2003) 438-44.
[55] J.R. Haynes, Influenza virus-like particle vaccines, Expert Review of Vaccines 8(4) (2009) 435-445.
[56] A. Naskalska, K. Pyrć, Virus Like Particles as Immunogens and Universal Nanocarriers, Pol. J. Microbiol. 64(1) (2015) 3-13.
[57] M. Tagliamonte, M.L. Tornesello, F.M. Buonaguro, L. Buonaguro, Chapter Eleven - Virus-Like Particles, in: M. Skwarczynski, I. Toth (Eds.), Micro and Nanotechnology in Vaccine Development, William Andrew Publishing2017, pp. 205-219.
[58] M.F. Bachmann, U.H. Rohrer, T.M. Kundig, K. Burki, H. Hengartner, R.M. Zinkernagel, The influence of antigen organization on B cell responsiveness, Science 262(5138) (1993) 1448-51.
[59] V. Cimica, J.M. Galarza, Adjuvant formulations for virus-like particle (VLP) based vaccines, Clinical immunology (Orlando, Fla.) 183 (2017) 99-108.
[60] N.M. Nour, Cervical cancer: a preventable death, Rev. Obstet. Gynecol. 2(4) (2009) 240-4.
[61] L. Jørgensen, P.C. Gøtzsche, T. Jefferson, Benefits and harms of the human papillomavirus (HPV) vaccines: systematic review with meta-analyses of trial data from clinical study reports, Systematic Reviews 9(1) (2020) 43.
[62] C. council, HPV vaccine, 2021. https://www.hpvvaccine.org.au/the-hpv-vaccine/has-the-program-been-successful.aspx.
[63] M. Tyler, E. Tumban, B. Chackerian, Second-generation prophylactic HPV vaccines: successes and challenges, Expert Review of Vaccines 13(2) (2014) 247-255.
[64] R. Gambhira, S. Jagu, B. Karanam, P.E. Gravitt, T.D. Culp, N.D. Christensen, R.B.S. Roden, Protection of Rabbits against Challenge with Rabbit Papillomaviruses by Immunization with the N Terminus of Human Papillomavirus Type 16 Minor Capsid Antigen L2, J. Virol. 81(21) (2007) 11585-11592.
[65] D.V. Pastrana, R. Gambhira, C.B. Buck, Y.-Y.S. Pang, C.D. Thompson, T.D. Culp, N.D. Christensen, D.R. Lowy, J.T. Schiller, R.B.S. Roden, Cross-neutralization of cutaneous and mucosal Papillomavirus types with anti-sera to the amino terminus of L2, Virology 337(2) (2005) 365-372.
[66] C.B. Pineo, I.I. Hitzeroth, E.P. Rybicki, Immunogenic assessment of plant-produced human papillomavirus type 16 L1/L2 chimaeras, Plant Biotechnol. J. 11(8) (2013) 964-975.
[67] B. Huber, C. Schellenbacher, C. Jindra, D. Fink, S. Shafti-Keramat, R. Kirnbauer, A Chimeric 18L1-45RG1 Virus-Like Particle Vaccine Cross-Protects against Oncogenic

Alpha-7 Human Papillomavirus Types, PLoS One 10(3) (2015) e0120152.
[68] K. Münger, B. Werness, N. Dyson, W. Phelps, E. Harlow, P. Howley, Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product, EMBO 8(13) (1989) 4099-4105.
[69] J. Martin Caballero, A. Garzón, L. González-Cintado, W. Kowalczyk, I. Jimenez Torres, G. Calderita, M. Rodriguez, V. Gondar, J.J. Bernal, C. Ardavín, D. Andreu, T. Zürcher, C. von Kobbe, Chimeric Infectious Bursal Disease Virus-Like Particles as Potent Vaccines for Eradication of Established HPV-16 E7–Dependent Tumors, PLoS One 7(12) (2013) e52976.
[70] S.F. Altekruse, J.S. Henley, J.E. Cucinelli, K.A. McGlynn, Changing Hepatocellular Carcinoma Incidence and Liver Cancer Mortality Rates in the United States, Am. J. Gastroenterol. 109(4) (2014).
[71] Y. Kim, A. Ejaz, A. Tayal, G. Spolverato, J.F.P. Bridges, R.A. Anders, T.M. Pawlik, Temporal trends in population-based death rates associated with chronic liver disease and liver cancer in the United States over the last 30 years, Cancer 120(19) (2014) 3058-3065.
[72] D. Lavanchy, Worldwide epidemiology of HBV infection, disease burden, and vaccine prevention, J. Clin. Virol. 34 (2005) S1-S3.
[73] K.N. Ly, J. Xing, R.M. Klevens, R.B. Jiles, J.W. Ward, S.D. Holmberg, The Increasing Burden of Mortality From Viral Hepatitis in the United States Between 1999 and 2007, Ann. Intern. Med. 156(4) (2012) 271-278.
[74] A. Cassidy, S. Mossman, A. Olivieri, M. De Ridder, G. Leroux-Roels, Hepatitis B vaccine effectiveness in the face of global HBV genotype diversity, Expert Rev Vaccines 10(12) (2011) 1709-15.
[75] M.R. Geier, D.A. Geier, A.C. Zahalsky, A review of hepatitis B vaccination, Expert Opin. Drug Saf. 2(2) (2003) 113-22.
[76] I. Sominskaya, D. Skrastina, I. Petrovskis, A. Dishlers, I. Berza, M. Mihailova, J. Jansons, I. Akopjana, I. Stahovska, D. Dreilina, A VLP Library of C-Terminally Truncated Hepatitis B Core Proteins: Correlation of RNA Encapsidation with a Th1/Th2 Switch in the Immune Responses of Mice, PLoS One 8(9) (2013) e75938.
[77] A. Dishlers, D. Skrastina, R. Renhofa, I. Petrovskis, V. Ose, I. Lieknina, J. Jansons, P. Pumpens, I. Sominskaya, The Hepatitis B Virus Core Variants that Expose Foreign C-Terminal Insertions on the Outer Surface of Virus-Like Particles, Mol. Biotechnol. 57(11-12) (2015) 1038-49.
[78] X. Ye, Z. Ku, Q. Liu, X. Wang, J. Shi, Y. Zhang, L. Kong, Y. Cong, Z. Huang, Chimeric Virus-Like Particle Vaccines Displaying Conserved Enterovirus 71 Epitopes Elicit Protective Neutralizing Antibodies in Mice through Divergent Mechanisms, J. Virol. 88(1) (2014) 72.
[79] J.K. Liu, Anti-cancer vaccines - a one-hit wonder?, Yale J Biol Med 87(4) (2014) 481-9.
[80] C.G. Drake, E.J. Lipson, J.R. Brahmer, Breathing new life into immunotherapy: review of melanoma, lung and kidney cancer, Nature reviews. Clinical oncology 11(1) (2014) 24-37.
[81] M. Wang, J. Zhao, L. Zhang, F. Wei, Y. Lian, Y. Wu, Z. Gong, S. Zhang, J. Zhou, K. Cao, X. Li, W. Xiong, G. Li, Z. Zeng, C. Guo, Role of tumor microenvironment in tumorigenesis, J. Cancer 8(5) (2017) 761-773.

[82] G.C. Cesana, G. DeRaffele, S. Cohen, D. Moroziewicz, J. Mitcham, J. Stoutenburg, K. Cheung, C. Hesdorffer, S. Kim-Schulze, H.L. Kaufman, Characterization of CD4+CD25+ Regulatory T Cells in Patients Treated With High-Dose Interleukin-2 for Metastatic Melanoma or Renal Cell Carcinoma, J. Clin. Oncol. 24(7) (2006) 1169-1177.

[83] D.-M. Kuang, Q. Zhao, C. Peng, J. Xu, J.-P. Zhang, C. Wu, L. Zheng, Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1, Journal of Experimental Medicine 206(6) (2009) 1327-1337.

[84] C.B. Williams, E.S. Yeh, A.C. Soloff, Tumor-associated macrophages: unwitting accomplices in breast cancer malignancy, npj Breast Cancer 2(1) (2016) 15025.

[85] K. Tegerstedt, J.A. Lindencrona, C. Curcio, K. Andreasson, C. Tullus, G. Forni, T. Dalianis, R. Kiessling, T. Ramqvist, A Single Vaccination with Polyomavirus VP1/VP2Her2 Virus-Like Particles Prevents Outgrowth of HER-2/neu–Expressing Tumors, Cancer Res. 65(13) (2005) 5953.

[86] J.S. Ross, J.A. Fletcher, G.P. Linette, J. Stec, E. Clark, M. Ayers, W.F. Symmans, L. Pusztai, K.J. Bloom, The HER-2/neu Gene and Protein in Breast Cancer 2003: Biomarker and Target of Therapy, The Oncologist 8(4) (2003) 307-325.

[87] J.T. Schiller, D.R. Lowy, Vaccines to prevent infections by oncoviruses, Annual review of microbiology 64 (2010) 23-41.

[88] P.M. Dimberu, R.M. Leonhardt, Cancer immunotherapy takes a multi-faceted approach to kick the immune system into gear, The Yale journal of biology and medicine 84(4) (2011) 371-380.

[89] P.J. Farrell, Epstein–Barr Virus and Cancer, Annual Review of Pathology: Mechanisms of Disease 14(1) (2019) 29-53.

[90] O.A. Odumade, K.A. Hogquist, H.H. Balfour, Progress and Problems in Understanding and Managing Primary Epstein-Barr Virus Infections, Clin. Microbiol. Rev. 24(1) (2011) 193-209.

[91] M.A. Epstein, B.G. Achong, Y.M. Barr, VIRUS PARTICLES IN CULTURED LYMPHOBLASTS FROM BURKITT'S LYMPHOMA, Lancet 1(7335) (1964) 702-3.

[92] D.G. van Zyl, J. Mautner, H.-J. Delecluse, Progress in EBV Vaccines, Front. Oncol. 9(104) (2019).

[93] A.E. Handel, A.J. Williamson, G. Disanto, L. Handunnetthi, G. Giovannoni, S.V. Ramagopalan, An Updated Meta-Analysis of Risk of Multiple Sclerosis following Infectious Mononucleosis, PLoS One 5(9) (2010) e12496.

[94] L.L. Laichalk, D. Hochberg, G.J. Babcock, R.B. Freeman, D.A. Thorley-Lawson, The dispersal of mucosal memory B cells: evidence from persistent EBV infection, Immunity 16(5) (2002) 745-54.

[95] S.P.L. Limited, 2020. https://www.sciencephoto.com/media/248222/view/tem-of-epstein-barr-virus. (Accessed March 2020).

[96] H. Hjalgrim, J. Friborg, M. Melbye, The epidemiology of EBV and its association with malignant disease, in: A. Arvin, G. Campadelli-Fiume, E. Mocarski, P.S. Moore, B. Roizman, R. Whitley, K. Yamanishi (Eds.), Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis, Cambridge University Press

[97] M.P. Thompson, R. Kurzrock, Epstein-Barr Virus and Cancer, Clinical Cancer Research

10(3) (2004) 803-821.
[98] R. Ruiss, S. Jochum, G. Wanner, G. Reisbach, W. Hammerschmidt, R. Zeidler, A Virus-Like Particle-Based Epstein-Barr Virus Vaccine, J. Virol. 85(24) (2011) 13105-13113.
[99] J.G. Ogembo, M.R. Muraswki, L.W. McGinnes, A. Parcharidou, R. Sutiwisesak, T. Tison, J. Avendano, D. Agnani, R.W. Finberg, T.G. Morrison, J.D. Fingeroth, A chimeric EBV gp350/220-based VLP replicates the virion B-cell attachment mechanism and elicits long-lasting neutralizing antibodies in mice, J. Transl. Med. 13(1) (2015) 50.
[100] G.S. Taylor, H.M. Long, J.M. Brooks, A.B. Rickinson, A.D. Hislop, The Immunology of Epstein-Barr Virus–Induced Disease, Annu. Rev. Immunol. 33(1) (2015) 787-821.
[101] G.S. Taylor, N.M. Steven, Therapeutic vaccination strategies to treat nasopharyngeal carcinoma, Chinese Clinical Oncology 5(2) (2016) 11.
[102] C. Gurer, T. Strowig, F. Brilot, M. Pack, C. Trumpfheller, F. Arrey, C.G. Park, R.M. Steinman, C. Münz, Targeting the nuclear antigen 1 of Epstein-Barr virus to the human endocytic receptor DEC-205 stimulates protective T-cell responses, Blood 112(4) (2008) 1231-1239.
[103] Centers for Disease Control and Prevention (CDC). https://www.cdc.gov/hepatitis/hcv/hcvfaq.htm.
[104] M.J. Alter, Epidemiology of viral hepatitis and HIV co-infection, J. Hepatol. 44(1 Suppl) (2006) S6-9.
[105] J.M. Micallef, J.M. Kaldor, G.J. Dore, Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies, Journal of Viral Hepatitis 13(1) (2006) 34-41.
[106] M.H. Somi, J. Etemadi, M. Ghojazadeh, S. Farhang, M. Faramarzi, S. Foroutan, M. Soleimanpour, Risk factors of HCV seroconversion in hemodialysis patients in tabriz, iran, Hepat. Mon. 14(6) (2014) e17417-e17417.
[107] P. Farci, H.J. Alter, S. Govindarajan, D.C. Wong, R. Engle, R.R. Lesniewski, I.K. Mushahwar, S.M. Desai, R.H. Miller, N. Ogata, Lack of protective immunity against reinfection with hepatitis C virus, Science 258(5079) (1992) 135-140.
[108] C.J. Tibbs, Methods of transmission of hepatitis C, J. Viral Hepat. 2(3) (1995) 113-9.
[109] P. Farci, H.J. Alter, A. Shimoda, S. Govindarajan, L.C. Cheung, J.C. Melpolder, R.A. Sacher, J.W. Shih, R.H. Purcell, Hepatitis C virus–associated fulminant hepatic failure, New England Journal of Medicine 335(9) (1996) 631-634.
[110] M. Kaito, S. Ishida, H. Tanaka, S. Horiike, N. Fujita, Y. Adachi, M. Kohara, M. Konishi, S. Watanabe, Morphology of hepatitis C and hepatitis B virus particles as detected by immunogold electron microscopy, Med. Mol. Morphol. 39(2) (2006) 63-71.
[111] E.-I. Pécheur, Lipoprotein Receptors and Lipid Enzymes in Hepatitis C Virus Entry and Early Steps of Infection, Scientifica 2012 (2012) 709853.
[112] S.-H. Jeong , M. Qiao, M. Nascimbeni, Z. Hu, B. Rehermann, K. Murthy, T.J. Liang, Immunization with Hepatitis C Virus-Like Particles Induces Humoral and Cellular Immune Responses in Nonhuman Primates, J. Virol. 78(13) (2004) 6995-7003.
[113] M. Lechmann, K. Murata, J. Satoi, J. Vergalla, T.F. Baumert, T.J. Liang, Hepatitis C virus–like particles induce virus-specific humoral and cellular immune responses in mice, Hepatology 34(2) (2001) 417-423.
[114] K. Murata, M. Lechmann, M. Qiao, T. Gunji, H.J. Alter, T.J. Liang, Immunization with

hepatitis C virus-like particles protects mice from recombinant hepatitis C virus-vaccinia infection, Proceedings of the National Academy of Sciences 100(11) (2003) 6753-6758.
[115] M. Qiao, K. Murata, A.R. Davis, S.-H. Jeong, T.J. Liang, Hepatitis C virus–like particles combined with novel adjuvant systems enhance virus-specific immune responses, Hepatology 37(1) (2003) 52-59.
[116] G.A. Elmowalid, M. Qiao, S.-H. Jeong, B.B. Borg, T.F. Baumert, R.K. Sapp, Z. Hu, K. Murthy, T.J. Liang, Immunization with hepatitis C virus-like particles results in control of hepatitis C virus infection in chimpanzees, Proceedings of the National Academy of Sciences 104(20) (2007) 8427-8432.
[117] P.T. Vietheer, I. Boo, H.E. Drummer, H.J. Netter, Immunizations with chimeric hepatitis B virus-like particles to induce potential anti-hepatitis C virus neutralizing antibodies, Antivir. Ther. 12(4) (2007) 477-87.
[118] S. Jahan, S. Khaliq, B. Ijaz, W. Ahmad, S. Hassan, Role of HCV Core gene of genotype 1a and 3a and host gene Cox-2 in HCV-induced pathogenesis, Virol J. 8(1) (2011) 155.
[119] S. Yutani, N. Komatsu, S. Shichijo, K. Yoshida, H. Takedatsu, M. Itou, R. Kuromatu, T. Ide, M. Tanaka, M. Sata, A. Yamada, K. Itoh, Phase I clinical study of a peptide vaccination for hepatitis C virus-infected patients with different human leukocyte antigen-class I-A alleles, Cancer Sci. 100(10) (2009) 1935-42.
[120] Y. Lei, J. Shao, F. Zhao, Y. Li, C. Lei, F. Ma, H. Chang, Y. Zhang, Artificially designed hepatitis B virus core particles composed of multiple epitopes of type A and O foot-and-mouth disease virus as a bivalent vaccine candidate, J. Med. Virol. 91(12) (2019) 2142-2152.
[121] J. Guo, A. Zhou, X. Sun, W. Sha, K. Ai, G. Pan, C. Zhou, H. Zhou, H. Cong, S. He, Immunogenicity of a Virus-Like-Particle Vaccine Containing Multiple Antigenic Epitopes of Toxoplasma gondii Against Acute and Chronic Toxoplasmosis in Mice, Front. Immunol. 10(592) (2019).
[122] S. Aston-Deaville, E. Carlsson, M. Saleem, A. Thistlethwaite, H. Chan, S. Maharjan, A. Facchetti, I.M. Feavers, C. Alistair Siebert, R.F. Collins, A. Roseman, J.P. Derrick, An assessment of the use of Hepatitis B Virus core protein virus-like particles to display heterologous antigens from Neisseria meningitidis, Vaccine 38(16) (2020) 3201-3209.
[123] A. Huang, R.N. de Jong, G.E. Folkers, R. Boelens, NMR characterization of foldedness for the production of E3 RING domains, J Struct Biol 172(1) (2010) 120-7.
[124] I. Sominskaya, D. Skrastina, A. Dislers, D. Vasiljev, M. Mihailova, V. Ose, D. Dreilina, P. Pumpens, Construction and immunological evaluation of multivalent hepatitis B virus (HBV) core virus-like particles carrying HBV and HCV epitopes, Clin. Vaccine Immunol. 17(6) (2010) 1027-33.
[125] W. Dai, P. Xiong, X. Zhang, Z. Liu, J. Chen, Y. Zhou, X. Ye, C. Zhang, Recombinant virus-like particle presenting a newly identified coxsackievirus A10 neutralization epitope induces protective immunity in mice, Antiviral Res. 164 (2019) 139-146.
[126] P. Liang, Y. Yi, Q.D. Su, F. Qiu, X.T. Fan, X.X. Lu, S.L. Bi, Efficient Humoral and Cellular Immune Responses Induced by a Chimeric Virus-like Particle Displaying the Epitope of EV71 without Adjuvant, Biomedical and Environmental Sciences 31(5) (2018) 343-350.
[127] A. Kazaks, I.N. Lu, S. Farinelle, A. Ramirez, V. Crescente, B. Blaha, O. Ogonah, T.

Mukhopadhyay, M.P. de Obanos, A. Krimer, I. Akopjana, J. Bogans, V. Ose, A. Kirsteina, T. Kazaka, N.J. Stonehouse, D.J. Rowlands, C.P. Muller, K. Tars, W.M. Rosenberg, Production and purification of chimeric HBc virus-like particles carrying influenza virus LAH domain as vaccine candidates, BMC Biotechnol. 17(1) (2017) 79.

[128] Y. Wu, R. Zhu, L. Xu, Y. Li, S. Li, H. Yu, S. Li, H. Zhu, T. Cheng, N. Xia, A novel combined vaccine based on monochimeric VLP co-displaying multiple conserved epitopes against enterovirus 71 and varicella-zoster virus, Vaccine 35(20) (2017) 2728-2735.

[129] N.N. Ninyio, K.L. Ho, H.K. Ong, C.Y. Yong, H.Y. Chee, M. Hamid, W.S. Tan, Immunological Analysis of the Hepatitis B Virus "a" Determinant Displayed on Chimeric Virus-Like Particles of Macrobrachium rosenbergii Nodavirus Capsid Protein Produced in Sf9 Cells, Vaccines 8(2) (2020) 275.

[130] X. Chen, T. Zhang, H. Liu, Y. Hao, G. Liao, X. Xu, Displaying 31RG-1 peptide on the surface of HPV16 L1 by use of a human papillomavirus chimeric virus-like particle induces cross-neutralizing antibody responses in mice, Hum. Vaccin. Immunother. 14(8) (2018) 2025-2033.

[131] R. Gonzalez-Castro, G. Acero Galindo, Y. García Salcedo, L. Uribe Campero, V. Vazquez Perez, M. Carrillo-Tripp, G. Gevorkian, M.A. Gomez Lim, Plant-based chimeric HPV-virus-like particles bearing amyloid-β epitopes elicit antibodies able to recognize amyloid plaques in APP-tg mouse and Alzheimer's disease brains, Inflammopharmacology 26(3) (2018) 817-827.

[132] D. Wang, S. Zhang, Y. Zou, W. Yu, Y. Jiang, Y. Zhan, N. Wang, Y. Dong, Y. Yang, Structure-Based Design of Porcine Circovirus Type 2 Chimeric VLPs (cVLPs) Displays Foreign Peptides on the Capsid Surface, Frontiers in Cellular and Infection Microbiology 8(232) (2018).

[133] M. Fraiberk, M. Hájková, M. Krulová, M. Kojzarová, A. Drda Morávková, I. Pšikal, J. Forstová, Exploitation of stable nanostructures based on the mouse polyomavirus for development of a recombinant vaccine against porcine circovirus 2, PLoS One 12(9) (2017) e0184870.

[134] L. He, J. Zhu, Computational tools for epitope vaccine design and evaluation, Curr. Opin. Virol. 11 (2015) 103-112.

[135] G. Roberta, Molecular Dynamics as a Tool in Rational Drug Design: Current Status and Some Major Applications, Curr. Comput. Aided Drug Des. 5(4) (2009) 225-240.

[136] W.L. Jorgensen, The Many Roles of Computation in Drug Discovery, Science 303(5665) (2004) 1813-1818.

[137] R. Twarock, Mathematical virology: a novel approach to the structure and assembly of viruses, Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences 364(1849) (2006) 3357-3373.

[138] S.A. Wynne, R.A. Crowther, A.G. Leslie, The crystal structure of the human hepatitis B virus capsid, Mol Cell 3(6) (1999) 771-80.

[139] X. Yu, L. Jin, J. Jih, C. Shih, Z.H. Zhou, 3.5Å cryoEM structure of hepatitis B virus core assembled from full-length core protein, PLoS One 8(9) (2013) e69729-e69729.

[140] J. Chen, C. Zhang, Y. Zhou, X. Zhang, C. Shen, X. Ye, W. Jiang, Z. Huang, Y. Cong, A 3.0-Angstrom Resolution Cryo-Electron Microscopy Structure and Antigenic Sites of

Coxsackievirus A6-Like Particles, J. Virol. 92(2) (2018) e01257-17.
[141] X. Mo, X. Li, B. Yin, J. Deng, K. Tian, A. Yuan, Structural roles of PCV2 capsid protein N-terminus in PCV2 particle assembly and identification of PCV2 type-specific neutralizing epitope, PLoS pathogens 15(3) (2019) e1007562-e1007562.
[142] B. Bishop, J. Dasgupta, M. Klein, R.L. Garcea, N.D. Christensen, R. Zhao, X.S. Chen, Crystal structures of four types of human papillomavirus L1 capsid proteins: understanding the specificity of neutralizing monoclonal antibodies, J Biol Chem 282(43) (2007) 31803-11.
[143] L. Zhang, L.H.L. Lua, A.P.J. Middelberg, Y. Sun, N.K. Connors, Biomolecular engineering of virus-like particles aided by computational chemistry methods, Chem. Soc. Rev. 44(23) (2015) 8608-8618.
[144] S.A. Adcock, J.A. McCammon, Molecular dynamics: survey of methods for simulating the activity of proteins, Chem Rev 106(5) (2006) 1589-615.
[145] M. Karplus, J.A. McCammon, Molecular dynamics simulations of biomolecules, Nat Struct Biol 9(9) (2002) 646-52.
[146] R. Zandi, D. Reguera, Mechanical properties of viral capsids, Phys. Rev. E Stat. Nonlin. Soft Matter Phys. 72(2 Pt 1) (2005) 021917.
[147] P.F. McMillan, D.C. Clary, D.J. Wales, The energy landscape as a unifying theme in molecular science, Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences 363(1827) (2005) 357-377.
[148] D.C. Rapaport, Molecular dynamics simulation of reversibly self-assembling shells in solution using trapezoidal particles, Physical Review E 86(5) (2012) 051917.
[149] D.C. Rapaport, Modeling capsid self-assembly: design and analysis, Physical Biology 7(4) (2010) 045001.
[150] M.F. Hagan, O.M. Elrad, R.L. Jack, Mechanisms of kinetic trapping in self-assembly and phase transformation, J. Chem. Phys. 135(10) (2011) 104115.
[151] J. Zheng, L. Zhang, R. Tang, S. Bai, N.K. Connors, L.H.L. Lua, Y.P. Chuan, A.P.J. Middelberg, Y. Sun, Energetic Changes Caused by Antigenic Module Insertion in a Virus-Like Particle Revealed by Experiment and Molecular Dynamics Simulations, PLoS One 9(9) (2014).
[152] R. Biabanikhankahdani, N.B.M. Alitheen, K.L. Ho, W.S. Tan, pH-responsive Virus-like Nanoparticles with Enhanced Tumour-targeting Ligands for Cancer Drug Delivery, Sci. Rep. 6(1) (2016) 37891.
[153] K. Jemon, V. Young, M. Wilson, S. McKee, V. Ward, M. Baird, S. Young, M. Hibma, An Enhanced Heterologous Virus-Like Particle for Human Papillomavirus Type 16 Tumour Immunotherapy, PLoS One 8(6) (2013) e66866.
[154] K.W. Lee, B.T. Tey, K.L. Ho, B.A. Tejo, W.S. Tan, Nanoglue: An Alternative Way To Display Cell-Internalizing Peptide at the Spikes of Hepatitis B Virus Core Nanoparticles for Cell-Targeting Delivery, Mol. Pharm. 9(9) (2012) 2415-2423.
[155] S.-M. Kang, M.-C. Kim, R.W. Compans, Virus-like particles as universal influenza vaccines, Expert review of vaccines 11(8) (2012) 995-1007.
[156] J.M. Patel, M.-C. Kim, V.F. Vartabedian, Y.-N. Lee, S. He, J.-M. Song, H.-J. Choi, S. Yamanaka, N. Amaram, A. Lukacher, C.D. Montemagno, R.W. Compans, S.-M. Kang, P. Selvaraj, Protein transfer-mediated surface engineering to adjuvantate virus-like

nanoparticles for enhanced anti-viral immune responses, Nanomed. Nanotechnol. Biol. Med. 11(5) (2015) 1097-1107.
[157] K.D. Brune, M. Howarth, New Routes and Opportunities for Modular Construction of Particulate Vaccines: Stick, Click, and Glue, Front. Immunol. 9 (2018) 1432-1432.
[158] A.F. Sander, P.L. Lollini, Virus-like antigen display for cancer vaccine development, what is the potential?, Expert Rev Vaccines 17(4) (2018) 285-288.
[159] S. Dai, H. Wang, F. Deng, Advances and challenges in enveloped virus-like particle (VLP)-based vaccines, J. Immunol. Sci 2(2) (2018) 36-41.
[160] J. Schumacher, T. Bacic, R. Staritzbichler, M. Daneschdar, T. Klamp, P. Arnold, S. Jagle, O. Tureci, J. Markl, U. Sahin, Enhanced stability of a chimeric hepatitis B core antigen virus-like-particle (HBcAg-VLP) by a C-terminal linker-hexahistidine-peptide, J Nanobiotechnology 16(1) (2018) 39.
[161] R. Russo, V.S. Panangala, R.R. Wood, P.H. Klesius, Chemical and electroporated transformation of Edwardsiella ictaluri using three different plasmids, FEMS Microbiol. Lett. 298(1) (2009) 105-110.
[162] T.F. Baumert, S. Ito, D.T. Wong, T.J. Liang, Hepatitis C virus structural proteins assemble into viruslike particles in insect cells, J. Virol. 72(5) (1998) 3827-3836.
[163] A. Zeltins, Construction and Characterization of Virus-Like Particles: A Review, Mol. Biotechnol. 53(1) (2013) 92-107.
[164] J. Fuenmayor, F. Gòdia, L. Cervera, Production of virus-like particles for vaccines, N. Biotechnol. 39(Pt B) (2017) 174-180.
[165] N.K. Jain, N. Sahni, O.S. Kumru, S.B. Joshi, D.B. Volkin, C. Russell Middaugh, Formulation and stabilization of recombinant protein based virus-like particle vaccines, Adv Drug Deliv Rev 93 (2015) 42-55.
[166] C.M. Mair, K. Ludwig, A. Herrmann, C. Sieben, Receptor binding and pH stability—how influenza A virus hemagglutinin affects host-specific virus infection, Biochimica et Biophysica Acta (BBA)-Biomembranes 1838(4) (2014) 1153-1168.
[167] K.A. Aires, A.M. Cianciarullo, S.M. Carneiro, L.L. Villa, E. Boccardo, G. Pérez-Martinez, I. Perez-Arellano, M.L. Oliveira, P.L. Ho, Production of human papillomavirus type 16 L1 virus-like particles by recombinant Lactobacillus casei cells, Appl Environ Microbiol 72(1) (2006) 745-52.
[168] M. Tan, W. Zhong, D. Song, S. Thornton, X. Jiang, E. coli-expressed recombinant norovirus capsid proteins maintain authentic antigenicity and receptor binding capability, J. Med. Virol. 74(4) (2004) 641-9.
[169] Y.-M. Hu, S.-J. Huang, K. Chu, T. Wu, Z.-Z. Wang, C.-L. Yang, J.-P. Cai, H.-M. Jiang, Y.-J. Wang, M. Guo, X.-H. Liu, H.-J. Huang, F.-C. Zhu, J. Zhang, N.-S. Xia, Safety of an Escherichia coli-expressed bivalent human papillomavirus (types 16 and 18) L1 virus-like particle vaccine: an open-label phase I clinical trial, Hum. Vaccin. Immunother. 10(2) (2014) 469-475.
[170] H.B. Bang, Y.H. Lee, Y.J. Lee, K.J. Jeong, High-Level Production of Human Papillomavirus (HPV) Type 16 L1 in Escherichia coli, J Microbiol Biotechnol 26(2) (2016) 356-63.
[171] A. Roldão, M.C. Mellado, L.R. Castilho, M.J. Carrondo, P.M. Alves, Virus-like particles in vaccine development, Expert Rev Vaccines 9(10) (2010) 1149-76.

[172] L. Xue, J. Liu, Q. Wang, C. Zhang, L. Xu, J. Luo, J. Wang, C. Qin, Y. Liu, Z. Su, Purification and assembling a fused capsid protein as an enterovirus 71 vaccine candidate from inclusion bodies to pentamer-based nanoparticles, Biochem. Eng. J. 117(Part A) (2017) 139-146.

[173] A.M. Murthy, Y. Ni, X. Meng, C. Zhang, Production and evaluation of virus-like particles displaying immunogenic epitopes of porcine reproductive and respiratory syndrome virus (PRRSV), Int. J. Mol. Sci. 16(4) (2015) 8382-96.

[174] L. Hou, H. Wu, L. Xu, F. Yang, Expression and self-assembly of virus-like particles of infectious hypodermal and hematopoietic necrosis virus in Escherichia coli, Arch Virol 154(4) (2009) 547-53.

[175] I. Kalnciema, D. Skrastina, V. Ose, P. Pumpens, A. Zeltins, Potato virus Y-like particles as a new carrier for the presentation of foreign protein stretches, Mol. Biotechnol. 52(2) (2012) 129-39.

[176] M. Mühlmann, E. Forsten, S. Noack, J. Büchs, Optimizing recombinant protein expression via automated induction profiling in microtiter plates at different temperatures, Microbial cell factories 16(1) (2017) 220-220.

[177] S.D. Brown, J.D. Fiedler, M.G. Finn, Assembly of hybrid bacteriophage Qbeta virus-like particles, Biochemistry 48(47) (2009) 11155-7.

[178] H. Guo, J. Zhu, Y. Tan, C. Li, Z. Chen, S. Sun, G. Liu, Self-assembly of virus-like particles of rabbit hemorrhagic disease virus capsid protein expressed in Escherichia coli and their immunogenicity in rabbits, Antiviral Res. 131 (2016) 85-91.

[179] S. Yin, S. Sun, S. Yang, Y. Shang, X. Cai, X. Liu, Self-assembly of virus-like particles of porcine circovirus type 2 capsid protein expressed from Escherichia coli, Virol J. 7 (2010) 166-166.

[180] S. Saraswat, T.N. Athmaram, M. Parida, A. Agarwal, A. Saha, P.K. Dash, Expression and Characterization of Yeast Derived Chikungunya Virus Like Particles (CHIK-VLPs) and Its Evaluation as a Potential Vaccine Candidate, PLoS Negl. Trop. Dis. 10(7) (2016) e0004782-e0004782.

[181] J. Freivalds, A. Dislers, V. Ose, D. Skrastina, I. Cielens, P. Pumpens, K. Sasnauskas, A. Kazaks, Assembly of bacteriophage Qbeta virus-like particles in yeast Saccharomyces cerevisiae and Pichia pastoris, J Biotechnol 123(3) (2006) 297-303.

[182] Y. Morikawa, T. Goto, D. Yasuoka, F. Momose, T. Matano, Defect of human immunodeficiency virus type 2 Gag assembly in Saccharomyces cerevisiae, J. Virol. 81(18) (2007) 9911-21.

[183] H. Lünsdorf, C. Gurramkonda, A. Adnan, N. Khanna, U. Rinas, Virus-like particle production with yeast: ultrastructural and immunocytochemical insights into Pichia pastoris producing high levels of the hepatitis B surface antigen, Microb Cell Fact 10 (2011) 48.

[184] T. Kato, V.K. Deo, E.Y. Park, Functional Virus-Like Particles Production Using Silkworm and Their Application in Life Science, Journal of Aquaculture & Research Development 3 (2012).

[185] X. Shen, D.L. Hacker, L. Baldi, F.M. Wurm, Virus-free transient protein production in Sf9 cells, Journal of Biotechnology 171 (2014) 61-70.

[186] J. Zhu, Mammalian cell protein expression for biopharmaceutical production, Biotechnol.

Adv. 30(5) (2012) 1158-1170.
[187] S. Buffin, I. Peubez, F. Barrière, M.-C. Nicolaï, T. Tapia, V. Dhir, E. Forma, N. Sève, I. Legastelois, Influenza A and B virus-like particles produced in mammalian cells are highly immunogenic and induce functional antibodies, Vaccine 37(46) (2019) 6857-6867.
[188] C.M. Thompson, E. Petiot, A. Mullick, M.G. Aucoin, O. Henry, A.A. Kamen, Critical assessment of influenza VLP production in Sf9 and HEK293 expression systems, BMC Biotechnol. 15(1) (2015) 31.
[189] K.P. Jayapal, K.F. Wlaschin, W. Hu, M.G. Yap, Recombinant protein therapeutics from CHO cells-20 years and counting, Chem. Eng. Prog. 103(10) (2007) 40.
[190] C. Li, F. Liu, M. Liang, Q. Zhang, X. Wang, T. Wang, J. Li, D. Li, Hantavirus-like particles generated in CHO cells induce specific immune responses in C57BL/6 mice, Vaccine 28(26) (2010) 4294-4300.
[191] D. Fontana, R. Kratje, M. Etcheverrigaray, C. Prieto, Immunogenic virus-like particles continuously expressed in mammalian cells as a veterinary rabies vaccine candidate, Vaccine 33(35) (2015) 4238-4246.
[192] L. Cervera, S. Gutiérrez-Granados, M. Martínez, J. Blanco, F. Gòdia, M.M. Segura, Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium, Journal of biotechnology 166(4) (2013) 152-165.
[193] C.M. Thompson, E. Petiot, A. Lennaertz, O. Henry, A.A. Kamen, Analytical technologies for influenza virus-like particle candidate vaccines: challenges and emerging approaches, Virol J. 10(1) (2013) 141.
[194] J.K. Ma, P.M. Drake, P. Christou, The production of recombinant pharmaceutical proteins in plants, Nat Rev Genet 4(10) (2003) 794-805.
[195] H.-L. Liu, W.-S. Li, T. Lei, J. Zheng, Z. Zhang, X.-F. Yan, Z.-Z. Wang, Y.-L. Wang, L.-S. Si, Expression of human papillomavirus type 16 L1 protein in transgenic tobacco plants, Acta biochimica et biophysica sinica 37(3) (2005) 153-158.
[196] M.A. D'Aoust, M.M.J. Couture, N. Charland, S. Trepanier, N. Landry, F. Ors, L.P. Vézina, The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza, Plant Biotechnol. J. 8(5) (2010) 607-619.
[197] R. Greco, M. Michel, D. Guetard, M. Cervantes-Gonzalez, N. Pelucchi, S. Wain-Hobson, F. Sala, M. Sala, Production of recombinant HIV-1/HBV virus-like particles in Nicotiana tabacum and Arabidopsis thaliana plants for a bivalent plant-based vaccine, Vaccine 25(49) (2007) 8228-8240.
[198] M. Aboud, M. Wolfson, Y. Hassan, M. Huleihel, Rapid purification of extracellular and intracellular Moloney murine leukemia virus, Archives of virology 71(3) (1982) 185-195.
[199] C. Peixoto, M. Sousa, A. Silva, M. Carrondo, P. Alves, Downstream processing of triple layered rotavirus like particles, Journal of biotechnology 127(3) (2007) 452-461.
[200] T. Vicente, C. Peixoto, M. Carrondo, P. Alves, Purification of recombinant baculoviruses for gene therapy using membrane processes, Gene Ther. 16(6) (2009) 766-775.
[201] M.C.M. Mellado, C. Peixoto, P.E. Cruz, M.J. Carrondo, P.M. Alves, Purification of recombinant rotavirus VP7 glycoprotein for the study of in vitro rotavirus-like particles assembly, J. Chromatogr. B 874(1-2) (2008) 89-94.

[202] E.V. Grgacic, D.A. Anderson, Virus-like particles: passport to immune recognition, Methods 40(1) (2006) 60-5.
[203] D. Skrastina, I. Petrovskis, R. Petraityte, I. Sominskaya, V. Ose, I. Lieknina, J. Bogans, K. Sasnauskas, P. Pumpens, Chimeric derivatives of hepatitis B virus core particles carrying major epitopes of the rubella virus E1 glycoprotein, Clinical and vaccine immunology : CVI 20(11) (2013) 1719-1728.
[204] J.U. Galula, G.-J.J. Chang, D.-Y. Chao, Production and Purification of Dengue Virus-like Particles from COS-1 Cells, Bio-protocol 9(12) (2019) e3280.
[205] L. Huhti, V. Blazevic, K. Nurminen, T. Koho, V.P. Hytönen, T. Vesikari, A comparison of methods for purification and concentration of norovirus GII-4 capsid virus-like particles, Archives of virology 155(11) (2010) 1855-1858.
[206] J. DelProposto, C.Y. Majmudar, J.L. Smith, W.C. Brown, Mocr: a novel fusion tag for enhancing solubility that is compatible with structural biology applications, Protein expression and purification 63(1) (2009) 40-49.
[207] Y.-C. Hu, C.-T. Tsai, Y.-C. Chung, J.-T. Lu, J.T.-A. Hsu, Generation of chimeric baculovirus with histidine-tags displayed on the envelope and its purification using immobilized metal affinity chromatography, Enzyme and microbial technology 33(4) (2003) 445-452.
[208] I. Fonda, M. Kenig, V. Gaberc-Porekar, P. Pristovaek, V. Menart, Attachment of histidine tags to recombinant tumor necrosis factor-alpha drastically changes its properties, TheScientificWorldJOURNAL 2 (2002).
[209] A. Goel, D. Colcher, J.-S. Koo, B.J. Booth, G. Pavlinkova, S.K. Batra, Relative position of the hexahistidine tag effects binding properties of a tumor-associated single-chain Fv construct, Biochimica et Biophysica Acta (BBA)-General Subjects 1523(1) (2000) 13-20.
[210] C. Peixoto, T.B. Ferreira, M.F. Sousa, M.J. Carrondo, P.M. Alves, Towards purification of adenoviral vectors based on membrane technology, Biotechnology progress 24(6) (2008) 1290-1296.
[211] R. Morenweiser, Downstream processing of viral vectors and vaccines, Gene Ther. 12(1) (2005) S103-S110.
[212] T. Vicente, M.F. Sousa, C. Peixoto, J.P. Mota, P.M. Alves, M.J. Carrondo, Anion-exchange membrane chromatography for purification of rotavirus-like particles, J. Membr. Sci. 311(1-2) (2008) 270-283.
[213] 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(n/a).
[214] Y. Yang, Y. Mengran, S. Zhang, G. Ma, Z. Su, Adsorption of virus-like particles on ion exchange surface: Conformational changes at different pH detected by dual polarization interferometry, Journal of Chromatography A 1408 (2015) 161-168.
[215] M. Yu, Y. Li, S. Zhang, X. Li, Y. Yang, Y. Chen, G. Ma, Z. Su, Improving stability of virus-like particles by ion-exchange chromatographic supports with large pore size: advantages of gigaporous media beyond enhanced binding capacity, J Chromatogr A 1331 (2014) 69-79.
[216] P. Kramberger, L. Urbas, A. Štrancar, Downstream processing and chromatography based analytical methods for production of vaccines, gene therapy vectors, and

bacteriophages, Hum. Vaccin. Immunother. 11(4) (2015) 1010-21.
[217] Vaccines, in: J.K. Aronson (Ed.), Meyler's Side Effects of Drugs (Sixteenth Edition), Elsevier, Oxford, 2016, pp. 255-293.
[218] L. Tomljenovic, C.A. Shaw, Aluminum vaccine adjuvants: are they safe?, Curr. Med. Chem. 18(17) (2011) 2630-7.
[219] E. Crisci, J. Bárcena, M. Montoya, Virus-like particles: The new frontier of vaccines for animal viral infections, Veterinary Immunology and Immunopathology 148(3) (2012) 211-225.
[220] A. Rynda-Apple, D.P. Patterson, T. Douglas, Virus-like particles as antigenic nanomaterials for inducing protective immune responses in the lung, Nanomedicine 9(12) (2014) 1857-1868.
[221] H. Li, Y. Yang, Y. Zhang, S. Zhang, Q. Zhao, Y. Zhu, X. Zou, M. Yu, G. Ma, Z. Su, A hydrophobic interaction chromatography strategy for purification of inactivated foot-and-mouth disease virus, Protein Expr. Purif. 113 (2015) 23-9.
[222] W. Zhou, J. Bi, L. Zhao, Y. Wang, Y. Li, Y. Huang, G. Ma, Z. Su, A highly efficient hydrophobic interaction chromatographic absorbent improved the purification of hepatitis B surface antigen (HBsAg) derived from Hansenula polymorpha cell, Process Biochem. 42(5) (2007) 751-756.
[223] P. Steppert, D. Burgstaller, M. Klausberger, A. Tover, E. Berger, A. Jungbauer, Quantification and characterization of virus-like particles by size-exclusion chromatography and nanoparticle tracking analysis, J Chromatogr A 1487 (2017) 89-99.
[224] J. Vajda, D. Weber, D. Brekel, B. Hundt, E. Müller, Size distribution analysis of influenza virus particles using size exclusion chromatography, J Chromatogr A 1465 (2016) 117-25.
[225] Y. Song, Y. Yang, Z. Su, L. Liu, Y. Zhu, Y. Xu, X. Zou, Q. Zhao, S. Zhang, [Vaccine pretreatment for quantification of 146S antigen in foot-and-mouth disease vaccines by high performance size exclusion chromatography], Sheng Wu Gong Cheng Xue Bao 35(8) (2019) 1441-1452.
[226] D. Bucafusco, S. Di Giacomo, J. Pega, J.M. Schammas, N. Cardoso, A.V. Capozzo, M. Perez-Filgueira, Foot-and-mouth disease vaccination induces cross-reactive IFN-γ responses in cattle that are dependent on the integrity of the 140S particles, Virology 476 (2015) 11-18.
[227] Y. Yang, H. Li, Z. Li, Y. Zhang, S. Zhang, Y. Chen, M. Yu, G. Ma, Z. Su, Size-exclusion HPLC provides a simple, rapid, and versatile alternative method for quality control of vaccines by characterizing the assembly of antigens, Vaccine 33(9) (2015) 1143-50.
[228] C. Ladd Effio, S.A. Oelmeier, J. Hubbuch, High-throughput characterization of virus-like particles by interlaced size-exclusion chromatography, Vaccine 34(10) (2016) 1259-67.
[229] P. Kondylis, C.J. Schlicksup, A. Zlotnick, S.C. Jacobson, Analytical Techniques to Characterize the Structure, Properties, and Assembly of Virus Capsids, Anal. Chem. 91(1) (2019) 622-636.
[230] P.W. Atkins, J. De Paula, Elements of physical chemistry, 2017.
[231] S.J. Hanslip, N.R. Zaccai, A.P. Middelberg, R.J. Falconer, Intrinsic fluorescence as an analytical probe of virus-like particle assembly and maturation, Biochem Biophys Res Commun 375(3) (2008) 351-5.

[232] A.B.T. Ghisaidoobe, S.J. Chung, Intrinsic tryptophan fluorescence in the detection and analysis of proteins: a focus on Förster resonance energy transfer techniques, Int. J. Mol. Sci. 15(12) (2014) 22518-22538.
[233] S. Ausar, T. Foubert, M. Hudson, T. Vedvick, C. Middaugh, Conformational stability and disassembly of Norwalk virus-like particles. Effect of pH and temperature. J Biol Chem 281(28):19478-88 (Epub 4 May 2006), The Journal of biological chemistry 281 (2006) 19478-88.
[234] S. Singh, A. Zlotnick, Observed Hysteresis of Virus Capsid Disassembly Is Implicit in Kinetic Models of Assembly, The Journal of biological chemistry 278 (2003) 18249-55.
[235] N.C. Garbett, G.N. Brock, Differential scanning calorimetry as a complementary diagnostic tool for the evaluation of biological samples, Biochim Biophys Acta 1860(5) (2016) 981-989.
[236] Z. Li, J. Wei, Y. Yang, L. Liu, G. Ma, S. Zhang, Z. Su, A two-step heat treatment of cell disruption supernatant enables efficient removal of host cell proteins before chromatographic purification of HBc particles, Journal of Chromatography A 1581-1582 (2018) 71-79.
[237] Z. Li, J. Wei, Y. Yang, X. Ma, B. Hou, W. An, Z. Hua, J. Zhang, Y. Li, G. Ma, S. Zhang, Z. Su, Strong hydrophobicity enables efficient purification of HBc VLPs displaying various antigen epitopes through hydrophobic interaction chromatography, Biochem. Eng. J. 140 (2018) 157-167.
[238] W. Jiang, L. Tang, Atomic cryo-EM structures of viruses, Curr. Opin. Struct. Biol. 46 (2017) 122-129.
[239] V.L. Popov, R.B. Tesh, S.C. Weaver, N. Vasilakis, Electron Microscopy in Discovery of Novel and Emerging Viruses from the Collection of the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA), Viruses 11(5) (2019).
[240] S. De Carlo, J.R. Harris, Negative staining and cryo-negative staining of macromolecules and viruses for TEM, Micron 42(2) (2011) 117-31.
[241] B. Metz, G. van den Dobbelsteen, C. van Els, J. van der Gun, L. Levels, L. van der Pol, N. Rots, G. Kersten, Quality-control issues and approaches in vaccine development, Expert Rev Vaccines 8(2) (2009) 227-38.

Chapter 3 Optimization of expression and purification of chimeric Hepatitis B core (HBc) VLPs

Abstract

Chimeric virus-like particle (VLP)-based vaccines were developed for the treatment of oncoviruses. In this chapter, two epitopes, Hepatitis C virus core (HCV core) and Epstein-Barr nuclear antigen 1 (EBNA1), targeting Hepatitis C virus (HCV) and Epstein–Barr Virus (EBV), were respectively fused into or Hepatitis B core (HBc) VLP protein at the N-terminus. The designed chimeric EBNA1-HBc VLP and HCV core-HBc proteins were expressed using *Escherichia coli* (*E. coli)* expression system. The expression conditions including cell density of *E. coli* for induction, expression temperature and concentration of inducer for HCV core-HBc VLP and EBNA1-HBc VLP were optimised to improve the yields of soluble expression and reduce the formation of inclusion body. The soluble expressed chimeric VLP proteins were precipitated using Ammonium Sulphate (AS) and acid precipitation to be separated from the majority of protein impurities. Different concentrations of AS and different pH values were used to find the optimal condition for the precipitation process. It was found that HCV core-HBc proteins were not stable after treating with acid and they were sensitive to the ion strength in the precipitation process. The yield after resuspension was influenced. AS precipitation, on the other hand, is promising for

removing impure proteins. Results indicate that 0.1 M AS was the optimal concentration for HCV core-HBc VLP precipitation and 1 M AS was optimal concentration for EBNA1-HBc VLP precipitation. Both HCV core-HBc VLP and EBNA1-HBc VLP achieved high production yields after optimization with 40.4 mg/g of wet cell weight for HCV core-HBc and 62.1 mg/g of wet cell weight for EBNA1-HBc. In addition, HCV core-HBc was chosen for the study of removal of nucleic acid impurities forming chimeric HBc VLP proteins. Results demonstrate that the nucleic acid impurities of HCV core-HBc VLP could be successfully removed using Poros HQ chromatography with flowthrough strategy. However, after the removal of nucleic acid impurities, HCV core-HBc VLP was found to be unstable in the storage condition. The study on structure and stability of chimeric HBc VLP proteins is necessary and is described in next chapter.

3.1. Introduction

Chimeric virus-like particle (VLP) based vaccine is a novel technology that generates vaccine candidates against oncoviruses which potentially lead to cancers[1]. To expand the application of VLP-based vaccines, chimeric VLPs, which were constructed by fusing foreign epitopes or antigens to well-studied VLPs, have been developed for the treatment of several viral infection diseases such as influenza[2, 3] and cancers[4-6]. Hepatitis B core (HBc) VLP is reported to be one of the most powerful VLP candidates to 1) display foreign epitopes for specific immunogenicity[7, 8], 2) expose cell-targeting signals[9, 10] and 3) package poly- and oligonucleotides[11]. Comparing with chemical coupling, antigens added through genetic modification is more controllable on, particularly, the antigen density and the insertion position. These modifications significantly influence the immunogenicity of chimeric VLPs. However, challenges of removal of host cell proteins (HCPs) and host cell DNA remain for the fused VLP proteins in their purification process. Therefore, rapid, efficient and cost-effective production process of chimeric HBc VLPs is needed for large scale production and future commercialization.

Previous works have proven that HBc VLP is able to display various foreign epitopes including HCC epitopes: MAGE-1, MAGE-3, AFP1 and AFP2[12], B cell epitopes of foot-and-mouth disease virus (FMDV)[13] and SP55 and SP70 epitope from EV71[14]. However, challenges remained, such as performing effective expression of chimeric HBc VLPs, removal of impurities and maintenance of assembled VLP structure after

purification. In addition, easily formation of mis-folding and aggregation was found after insertion of epitope to HBc VLP[15, 16]. These challenges can lead to failure to induce epitope specific immune response[17].

Different eukaryotic and prokaryotic expression systems were employed to produce chimeric VLP vaccines. Eukaryotic expression systems such as insect cells and mammalian cells obtain better post-translation modification systems and can be employed to express some structurally complicated and enveloped VLPs[18]. However, eukaryotic expression system is often regarded as high cost production because it requires long culture period and the expensive media[19]. In addition, reports also indicate that the glycosylation in eukaryotic expression system may cause improper modification of epitopes on the expressed proteins[20]. Prokaryotic expression system such as bacteria expression system, on the other hand, is regarded as low-cost approach with high expression yield in the production of recombinant proteins. In the production of VLPs, due to the lack of post-translation modification, bacteria expression system is commonly employed for the expression of non-enveloped and structurally simple VLPs such as HBc VLPs[21, 22]. The production yield of VLPs in bacteria expression system is higher and production time of bacteria expression system is less compared with eukaryotic expression system[23]. However, reports have indicated that VLPs expressed by bacteria expression system may be expressed as insoluble form such as inclusion bodies. This increases the difficulty in the downstream purification as the refolding process is required[13, 14, 24]. To improve the soluble expression of chimeric

VLP proteins in bacteria expression system, several approaches have been adopted including 1) control of expression conditions during the expression including temperature, inducer concentration and density of bacteria for expression[25], 2) selection of an appropriate expression plasmid with a suitable resistance marker[26], 3) fusion of a tagged protein such as glutathione-S-transferase (GST)[27] and small ubiquitin-like modifier protein (SUMO)[28].

To match the regulation for commercial VLP vaccines, effective downstream purification process is needed to remove the impurities including proteins and nucleic acids from host cells. Different from other recombinant proteins, purification of VLP proteins has the challenges to maintain stable VLP structure which is directly related to its immunogenicity after the purification process[29, 30]. Various purification methods have been developed according to the property of VLPs. For example, ultracentrifugation was applied to obtain pure VLP proteins rapidly. However, the yield of this approach is relatively low, and the cost is high. AS precipitation has been reported to obtain good purification results in HBc VLP[31] and HPV VLP[32] as it has a minor impact on the VLP structure. However, the optimal condition for AS precipitation may vary according to different cases to avoid the co-precipitation with impure proteins from host cells[33]. Acid precipitation is another promising approach to remove impure proteins[34]. It relies on the different stability of target VLP and impurities in lower pH values. In addition, chromatography is one popular method for the purification and polishing of VLP proteins[35, 36]. However, researchers indicated

that changes in microenvironments due to different buffer condition, the interactions between VLPs and resin and harsh operating conditions such as extreme pH and high salt concentration can lead to the aggregation or disassembly of target VLPs[37]. In this case, different approaches for purification of new chimeric VLP proteins need to be examined.

In our study, two epitopes, two different epitopes, Epstein-Barr nuclear antigens 1 (EBNA1), and Hepatitis C virus (HCV) core, were selected to fuse to the N-terminus of HBc VLP to form chimeric EBNA1-HBc VLP and HCV core-HBc VLP. EBNA1 and HCV core were reported as two epitopes targeting to Epstein-Barr virus (EBV) and Hepatitis C virus (HCV) infection respectively leading to cancers. EBNA1 epitope was chosen as the representative of short and non-structural epitope while HCV core was chosen as long and structural epitope for the study of impact of insertion of different epitopes on the purification of chimeric HBC VLPs. The recombinant HCV core-HBc and EBNA1-HBc proteins were expressed in *E. coli* expression system and the optimization of expression was conducted. After expression, different concentrations of AS were used to find the optimal one for the purification of HCV core-HBc and EBNA1-HBc proteins. Ion exchange chromatography was employed to remove the nucleic acids in the protein samples.

3.2. Materials and methods

3.2.1 Plasmids construction for recombinant HCV core-HBc and EBNA1-HBc

Figure 3.1 demonstrates the construction of the plasmids of the chimeric VLPs used in this study. The hepatitis B core antigen (HBc) gene within pET 21a(+)-HBc (State Key Laboratory of Biochemical Engineering of Institute of Process Engineering (IPE) of Chinese Academy of Sciences (CAS), China) was digested by XhoI/NdeI and then ligated into pET 30a plasmid (IPE of CAS, China) to generate an intermediate plasmid pET 30a-HBc. To make the HCV core (aa 10-53: KTKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRATRKTS) fused HBc protein, two steps of polymerase chain reactions (PCRs) were performed. Briefly, in the first step, two complementary oligonucleotides HCV core-F, GGAATTCCATATGAAAACCAAAAGAAACACA and HCV core-R, ACCGAACTCTTTGTATGGGTCGATGTCCATAC T were designed and used to amplify the HCV core sequence from the template supplied from Beijing Genomics Institute, China. Then another two complementary oligonucleotides HBc-F, ATGGACATCGACCCATACAAAGAGTTCGGT and HBc-R, CCGCTCGAGTTAA CACTGAGATTCACG were applied to amplify the HBc sequence from pET 30a-HBc template. In the second step, HCV core-F, GGAATTCCATATGAAAACCAAA AGAAACACA and HBc-R, CCGCTCGAGTTAACACTGAGATTCACG were used for an overlapped PCR to obtain HCV core-HBc gene sequence. The obtained HCV

core-HBc gene sequence was then ligated into pET 30a plasmid to yield pET 30a-HCV core-HBc plasmid. Plasmid pET 30a- EBNA1 (aa 407-417: HPVGEADYFEY)-HBc was supplied by Beijing Genomics Institute, China. The obtained plasmid sequence was confirmed by gene sequencing by Beijing Genomics Institute, China.

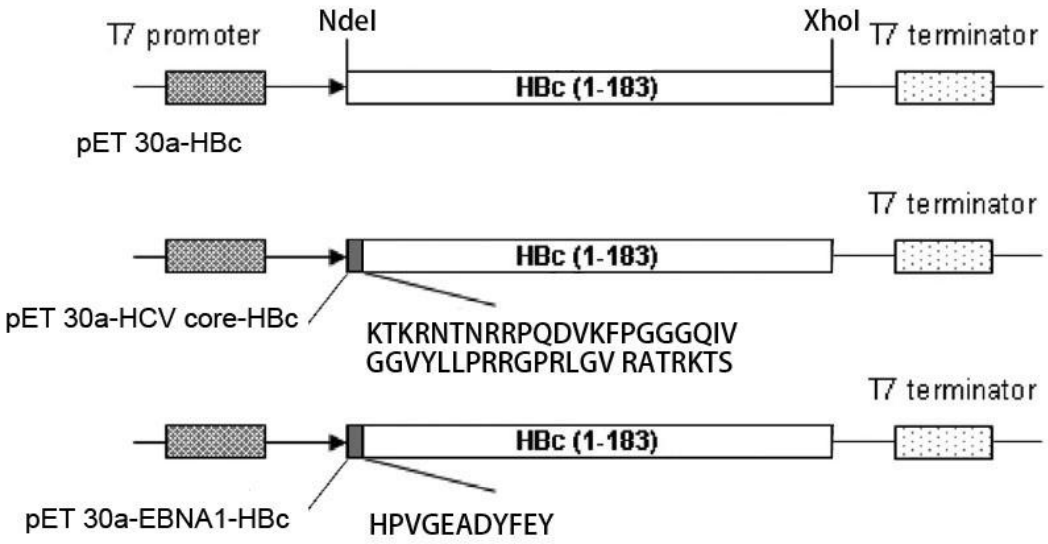

Figure 3.1 Schematic representation of the expression cassettes of the pET 30a-HBc, pET 30a-HCV core-HBc and pET 30a-EBNA1-HBc.

3.2.2 Expression of chimeric VLPs in *E. coli* expression system

After confirming the plasmid sequences of chimeric HBc VLPs, the plasmids were transferred to *E. coli* BL21 (DE3) strain (Thermo Fisher Scientific, USA) for the expression in Erlenmeyer flask in the shaker. pET 30a-HCV core-HBc and pET EBNA1-HBc were transferred to *E. coli* competent cells and grow on Luria-Bertani (LB) agarose plate overnight at 37 °C. Then, a single colony of HCV core-HBc and EBNA1 was cultured in 5 ml of LB medium (Thermo Fisher Scientific, USA) supplemented with 100 μg/ml kanamycin (Thermo Fisher Scientific, USA) at 37 °C overnight, respectively. 50 μl of precultured chimeric HBc BL21 *E. coli* was cultured

in 50 ml LB medium supplemented with 100 μg/ml kanamycin at 37 °C overnight. *E. coli* BL21 cells containing HCV core-HBc and EBNA1-HBc plasmid were stored in 25 % glycerol at -70 ℃ for future use after preculture. Target HCV core-HBc and EBNA1-HBc proteins were expected to be expressed in soluble form. Initially (No. 1 in **Table 3.1**), the expression conditions of HCV core-HBc and EBNA1-HBc followed the previous work of wt HBc VLP in IPE-CAS[38]. Briefly, the precultured chimeric HBc *E. coli* cells were transferred to 2 L LB medium with 100 μg/ml kanamycin in the ratio of 1:1000 and cultured at 37.5 ℃ for 4 h at 220 rpm. 1 mM of isopropyl b-thiogalactoside (IPTG) (Thermo Fisher Scientific, USA) was added for the expression of HCV core-HBc and EBNA1-HBc proteins. The culture was then incubated for 4 h at 37 °C with 180 rpm.

Then the expression condition was optimized using control variable approach according to **Table 3.1**. Three factors including 1) induction cell density of bacteria, 2) expression temperature and 3) the concentration of IPTG were examined orderly, and the better condition was applied in the next expression.

Table 3.1 Optimization of expression conditions of HCV core-HBc including concentration of IPTG, expression temperature and cell density of *E. coli* when adding IPTG.

No	Concentration of IPTG	Temperature	Cell density (OD_{600})
1	1 mM	37 ℃	1.2
2	1 mM	37 ℃	0.8
3	1 mM	30 ℃	0.8
4	0.5 mM	37 ℃	0.8

The cells were then harvested by centrifugation at 4,000 rpm for 20 min at 4 °C. The pellets were resuspended with 100 ml lysate buffer (20 mM Tris-HCl, 3 mM EDTA, 1 mM Phenylmethanesulfonylfluoride (PMSF), 0.1 % Triton X100, pH 8.0). Ultrasonic homogenizer (Scientz-IID, Ningbo Scientz Bio-Tech Co, Ltd, China) with a 4-s on and 6-s off pulse was used for the disruption of *E. coli* cells for 10 min at 360W. The crude lysate was then centrifuged at 10,000 rpm for 30 min. The supernatant and pellets after centrifugation together with the crude lysate before centrifugation were loaded to 12 % reducing Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described in **section 3.2.6** for evaluation later. The concentrations of protein samples were examined using Bradford assay[39]. The supernatant of bacteria lysate of EBNA1-HBc and HCV core-HBc was observed using Transmission Electron Microscopy (TEM) as described in **section 3.2.7** to examine the structure of expressed chimeric HBc VLPs.

3.2.3 AS precipitation of chimeric VLPs

Ammonium Sulphate (AS) precipitation was applied to purify HCV core-HBc and EBNA1-HBc proteins to avoid influences such as the disassembly and damage on their VLP structure, and high purity and recovery yield were expected. In the first trial, AS precipitation condition for wt HBc VLP in previous work in IPE-CAS[38] was applied for purification of HCV core-HBc and EBNA1-HBc proteins. Briefly, 1 M AS was added to the crude lysate of HCV core-HBc and EBNA1-HBc samples. Then the mixture was centrifuged at 6,000 rpm for 10 min. The mixture was stirred for 30 min

at room temperature. The pellet was resuspended using 4 M urea buffer (50 mM Glycine-NaOH with 4 M urea , pH 9). Resuspended samples were centrifuged at 10,000 rpm for 30 min. Supernatants and pellets in the precipitation step and resuspension step were loaded to 12 % reducing SDS-PAGE as described in **section 3.2.6** for examination. After reviewing the result of first trial of the AS precipitation of HCV core-HBc and EBNA1-HBc proteins, optimization of resuspension buffer and concentration of AS for HCV core-HBc was conducted.

3.2.3.1 Optimization of resuspension buffer for HCV core-HBc

To optimize the resuspension buffer for HCV core-HBc, two factors including pH value of buffer and concentration of urea were evaluated using control variable method. Briefly, 4 M urea buffers in different pH values including pH 10, pH 9, pH 8 and pH 7 were firstly applied to resuspend HCV core-HBc proteins after precipitation with 1 M AS. The resuspended samples were then centrifuged at 10,000 rpm for 30 min.

After choosing the optimal pH value, different concentrations of urea were applied. 4 M urea buffer, 6 M urea and 8 M urea buffer (50 mM Glycine-NaOH with 6 M urea , pH 9 and 50 mM Glycine-NaOH with 8 M urea) were used to resuspend HCV core-HBc proteins after 1 M AS precipitation. The resuspended samples were then centrifuged at 10,000 rpm for 30 min. The concentrations of supernatants of resuspended samples were measured using Bradford assay and recovery yields were calculated using equation 3.1 for analysis. In addition, Transmission Electron Microscopy (TEM) as described in **section 3.2.7** was employed to evaluate whether

high concentration of urea would impact on the structure of HCV core-HBc. After analysis, the optimal resuspension buffer for AS precipitation was employed in the following optimization of AS concentration.

3.2.3.2 Optimization of AS concentration for HCV core-HBc

To optimize the AS concentration for HCV core-HBc AS precipitation process, different concentrations of AS including 1 M, 0.5 M, 0.1 M, 0.05 M and 0.01 M were tested. The pellets of each condition were then collected and resuspended using 4 M urea buffer (50 mM Glycine-NaOH with 4 M urea , pH 9) which was the optimal resuspension buffer in previous test. Resuspended samples were centrifuged at 10,000 rpm for 30 min. The concentrations of supernatants of resuspended samples were measured using Bradford assay and recovery yields were calculated using equation 3.1 for analysis. Supernatants and pellets in the precipitation step and resuspension step were loaded to SDS-PAGE as described in **section 3.2.6** for examination. Supernatant samples in resuspension step were imaged using TEM as described in **section 3.2.7**.

3.2.4 Acid precipitation for chimeric HCV core-HBc

In the first trial of AS precipitation for HCV core-HBc protein, low recovery yield was achieved. In this case, another purification process for tag-free HBc VLP, acid precipitation was applied. Briefly, the supernatant of HCV core-HBc crude lysate was collected. Then pH value was adjusted using buffers including 100 mM Phosphate buffer (PB), pH7.0, pH 6.5 and pH 6.0 and 100 mM Acetic acid/Sodium acetate buffer (Acetate buffer), pH 5.5, pH 5.0 and pH 4.5 to perform a mild and steady change of pH.

The precipitated samples were then centrifuged at 6000 rpm for 10 min. 4 M urea buffer was used to resuspend the pellets. The resuspended sample was then centrifuged at 10,000 rpm for 30 min. The concentrations of supernatants of resuspended samples were measured using Bradford assay and recovery yield was calculated using equation 3.1 for analysis. The supernatant after acid precipitation and supernatant after resuspension were loaded to 12 % reducing SDS-PAGE as described in **section 3.2.6** for evaluation. The resuspension samples precipitated at pH 6.5 and pH 6.0 were imaged using TEM as described in **section 3.2.7**.

3.2.5 Removal of nucleic acids in chimeric HBc VLPs using chromatography

After optimization of AS and acid precipitation, the impure proteins from host cells were removed. However, nucleic acid impurities from host cells still existed. In this chapter, ion exchange chromatography was applied to remove these impurities. HCV core-HBc proteins was used for the preliminary study of the optimal conditions for removal of nucleic acid impurities.

3.2.5.1 Binding strategy for cationic exchange chromatography

Initially, binding strategy of ion exchange chromatography was designed. The theoretical isoelectric point (pI) of HCV core-HBc is 11.03 according to its amino acid sequence and the buffer pH after AS precipitation process was pH 9. In theory, HCV core-HBc should obtain positive charge. Based on this, two cationic exchange chromatography (CEC), HiTrap CM Sepharose FF and HiTrap SP Sepharose FF

(Cytiva, USA) chromatography were employed using AKTA pure system (Cytiva, USA). 50 mM Tris-HCl, 4 M urea , pH 9 was used as equilibration buffer since precipitation of HCV core-HBc was observed when using other buffering system such as PB. 50 mM Tris-HCl, 4 M urea , 1 M NaCl, pH 9 was used as elution buffer.

3.2.5.2 Flowthrough strategy for anionic exchange chromatography

After analysis of the result in previous binding strategy for removal of nucleic acids, the acquired results were not desirable, and HCV core-HBc failed to bind with the cationic exchange column in all tested conditions. Therefore, a flowthrough strategy using anionic exchange chromatography to separate HCV core-HBc proteins from nucleic acid impurities was designed. Briefly, three anionic exchange chromatography (AEC), Capto™ Q chromatography, HiTrap® Q chromatography and POROS™ 50 HQ chromatography (Cytiva, USA) were applied using AKTA pure system (Cytiva, USA). For Capto™ Q chromatography and HiTrap® Q Fast Flow (FF) chromatography, protein samples after resuspension were loaded to Capto™ Q, HiTrap® Q FF and POROS™ 50 HQ chromatography using 20 mM Tris-HCl, 4 M urea , pH 9 as equilibration buffer and 20 mM Tris-HCl, 4 M urea , 1 M NaCl, pH 9 as elution buffer. Isocratic elution was performed as HCV core-HBc VLPs should obtain positive charge and nucleic acids should obtain negative charge when pH is 9, in theory. Flowthrough (FT) fraction and elution fraction were collected. All samples in different fractions were analysed by 12 % reducing SDS-PAGE as described in **section 3.2.6** and agarose gel electrophoresis to examine the proteins and nucleic acids, respectively.

3.2.6 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate the protein samples according to their molecular weights and to evaluate the purity of target protein in samples [32]. In SDS-PAGE analysis, 12 % SDS polyacrylamide gel was employed. HCV core-HBc and EBNA1-HBc protein samples (5 μg per lane) were mixed with SDS-gel electrophoresis sample buffer containing 5 % SDS and 5 % 2-mercaptoethanol. Samples were heated at 100 ºC for 10 min before being loaded to the SDS-PAGE gel. 90 V of power was used for the stacking gel and 120 V of power was used for the separating gel. The proteins samples were separated on 12 % SDS polyacrylamide gel in vertical chambers (Bio-Rad, USA).

3.2.7 Transmission Electron Microscopy (TEM)

Expressed crude lysates and purified samples of HCV core-HBc and EBNA1-HBc were visualized with TEM. Briefly, 5 μl sample at concentration of 0.5 mg/ml was dipped and incubated the carbon-Formvar coated copper grids (Zhongjingkeyi Technology, China) on the surface for 10 min and touched dry on blotting paper. Then the grid was dipped and negatively stained with 1% uranyl acetate aqueous solution. The grids were examined according to the instructions of the TEM microscope (Hitachi JEM-1400, Japan), following the local guidelines.

3.2.8 Measurement of protein concentration and purity and purification yield determination

The concentrations of protein samples were examined using Bradford assay according

to the manufacturer's instruction. The purity of target protein was estimated according to the intensity calculation using SDS-PAGE image using ImageJ[40] and recovery yield was calculated using the following equation:

$$Revovery\ yield = \frac{c_{supernatant\ after\ resuspension} \times Purity\ after\ purification\ (\%)}{c_{total\ soluble\ proteins} \times Purity\ before\ purification\ (\%)} \times 100\% \quad \textbf{(Eq. 3.1)}$$

The production yields of chimeric HBc VLPs were calculated using the following equation:

$$Production\ yield = \frac{c_{supernatatant\ after\ dialysis} \times Purity\ of\ target\ protein\ (\%)}{mass\ (g)_{wet\ biomass}\ or\ volume(L)_{biomass}} \times 100\% \quad \textbf{(Eq. 3.2)}$$

3.3 Results

3.3.1 Plasmid construction and optimization of expression of chimeric VLP in *E. coli* expression system

The N-terminal of HBc was exposed on the surface of VLP structure and can be inserted with large epitopes without any impact on the VLP assembly[41]. Therefore, structural long epitope, HCV core epitope (aa 10-53: KTKRNTNRRPQDVKFPGGGQ IVGGVYLLPRRGPRLGVRATRKTS) and relatively short EBNA1 (aa 407-417: HPVGEADYFEY) were designed to be fused at N-terminal of HBc to form the chimeric HCV core-HBc VLP and EBNA1-HBc VLP (as shown in **Figure 3.1**). pET 30a HCV core-HBc plasmid was generated using Overlap Extension PCR[42]. Constructed gene sequence of HCV core-HBc was confirmed by gene sequencing(data not shown). Confirmed pET 30a-HCV core-HBc plasmid was transformed into *E. coli* BL21 (DE) strain for expression of HCV core-HBc proteins. Constructed pET 30a-EBNA1-HBc plasmid was supplied by Beijing Genomics Institute, China and pET 30a-

EBNA1-HBc plasmid was also transformed to *E. coli* BL21 (DE) strain for expression.

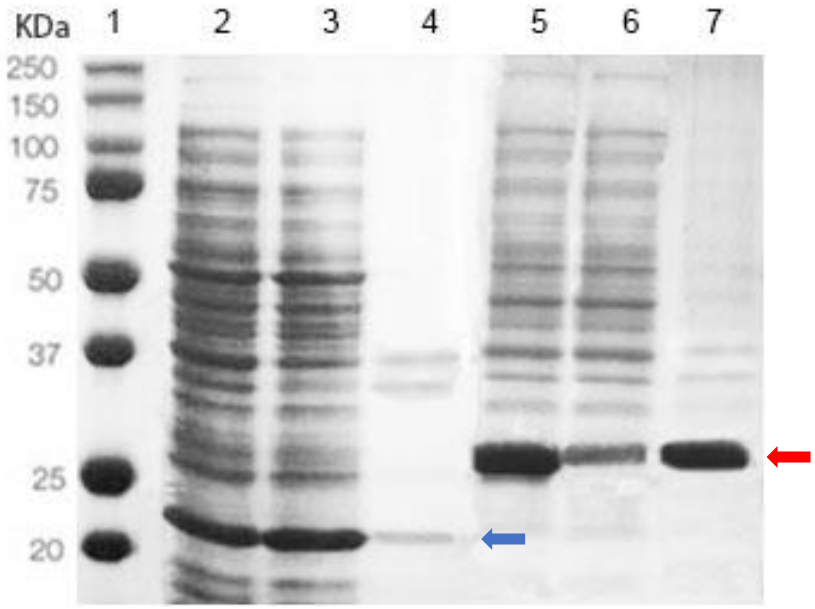

Figure 3.2 SDS-PAGE of expression of EBNA1-HBc (Lane 2-4) and HCV core-HBc (Lane 5-7) using expression condition of wt HBc VLP. Lane 1: molecular weight marker; Lane 2 and 5: crude lysate; Lane 3 and 6: supernatant of crude lysate of EBNA1-HBc; Lane 4 and 7: pellets of crude lysate. (red arrow: HCV core-HBc, blue arrow: EBNA1-HBc)

For the first trial for the expression of HCV core-HBc and EBNA1-HBc, expression conditions followed the expression of wt HBc VLP. As shown in **Figure 3.2**, around 97 % target EBNA1-HBc proteins (according to the intensity calculation using SDS-PAGE image) were expressed in the soluble part of *E. coli* while around 80 % of HCV core-HBc proteins were expressed in the inclusion bodies of *E. coli*. Therefore, optimization for expression of HCV core-HBc was conducted. Following the plan as described in **section 3.2.2**, three runs of expression was performed orderly. The harvested bacteria cells were then disrupted using ultrasonic homogenizer and centrifuged. The crude lysate together with supernatant and pellet after centrifugation were examined using 12 % reducing SDS-PAGE.

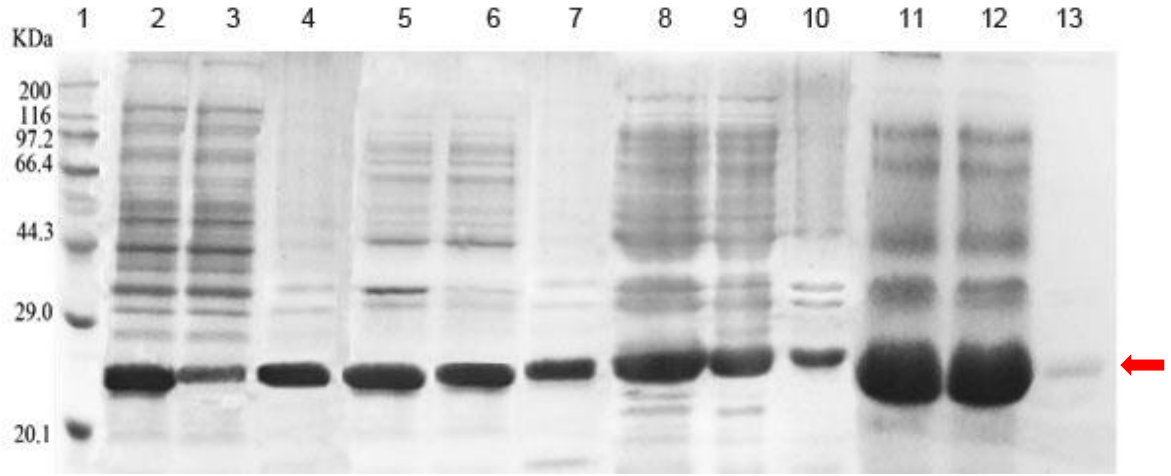

Figure 3.3 SDS-PAGE of optimization of expression of HCV core-HBc following **Table 3.1**, No.1: Lane 2-4, No.2: Lane 5-7, No.3: Lane 8-10, No.4: Lane 11-13. Lane 1: molecular weight marker; Lane 2,5,8 and 11: crude lysate of HCV core-HBc; Lane 3,6,9 and 12: supernatant of crude lysate of HCV core-HBc; Lane 4,7,10 and 13: pellets of crude lysate of HCV core-HBc. (red arrow: HCV core-HBc)

As shown in **Figure 3.3**, all SDS-PAGE results for these four runs were merged for analysis. For induction cell density, the expressed soluble form of HCV core-HBc proteins reached to 54 % with lower induction cell density of OD_{600} at 0.8, therefore, OD_{600} at 0.8 was chosen for next expression. For expression temperature evaluation, there is no significant difference of the percentage of soluble HCV core-HBc proteins between two expression temperature and 37 ℃ was chosen for the next expression as *E. coli* had a lower growth rate at 30 ℃, leading to less amount of biomass when the same expression time was applied. For concentration of IPTG, more than 95 % of expressed HCV core-HBc proteins were in the soluble form with the addition of 0.5 mM IPTG. With these results, the optimal soluble expression of HCV core-HBc proteins is to be expressed with induction of 0.5 mM IPTG when OD_{600} is 0.8 and expression temperature is 37 ℃. TEM images of soluble part of *E. coli* of HCV core-HBc and EBNA1-HBc demonstrate that both chimeric HBc proteins were expressed in

clear VLP structure (shown in **Figure 3.4**). The expressed HCV core-HBc and EBNA1-HBc proteins were then purified with AS precipitation.

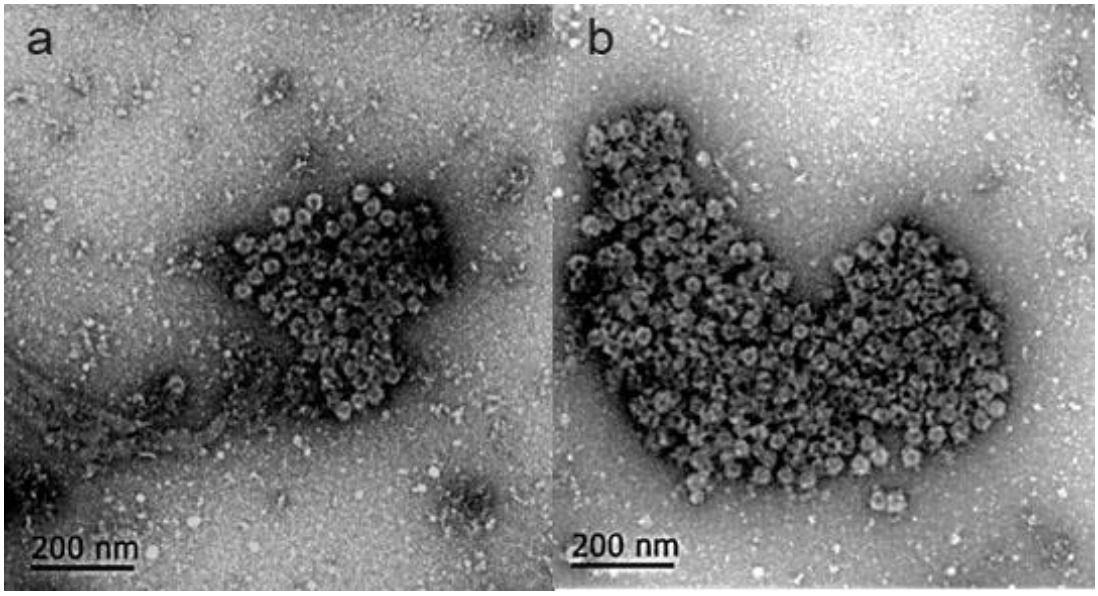

Figure 3.4 TEM image of HCV core-HBc protein (a) and EBNA1-HBc protein (b) in the soluble part of bacteria lysate

3.3.2 Purification of chimeric HBc VLPs

According to the purification process of HBc VLP described by the research group in IPE[43], ammonium sulphate (AS) precipitation was applied. 1 M AS was used for precipitation step and 4 M urea buffer, pH 9 was employed as the resuspension buffer. In the trial of AS precipitation for HCV core-HBc and EBNA1-HBc was found that EBNA1-HBc achieved a good recovery yield using the same AS concentration and resuspension buffer as wt HBc VLP. After 1 M AS precipitation and 4 M urea buffer resuspension, the purity of EBNA1-HBc reached 95 % and the recovery yield for AS precipitation process was more than 39.1% (target protein/total protein including impurities) while the recovery yield of HCV core-HBc was only 5 % (shown in **Figure 3.5**). After being dialysed against 20 mM Tris-HCl buffer, pH 7.4, the production yield

of EBNA1-HBc protein was determined, which was around 62.1 mg/g wet cell weight or 248.4 mg/L. Therefore, the study focused on the optimization of HCV core-HBc proteins as the recovery yield of HCV core-HBc in the trial was only around 5 %. Most of the target HCV core-HBc remained in the pellet after resuspension.

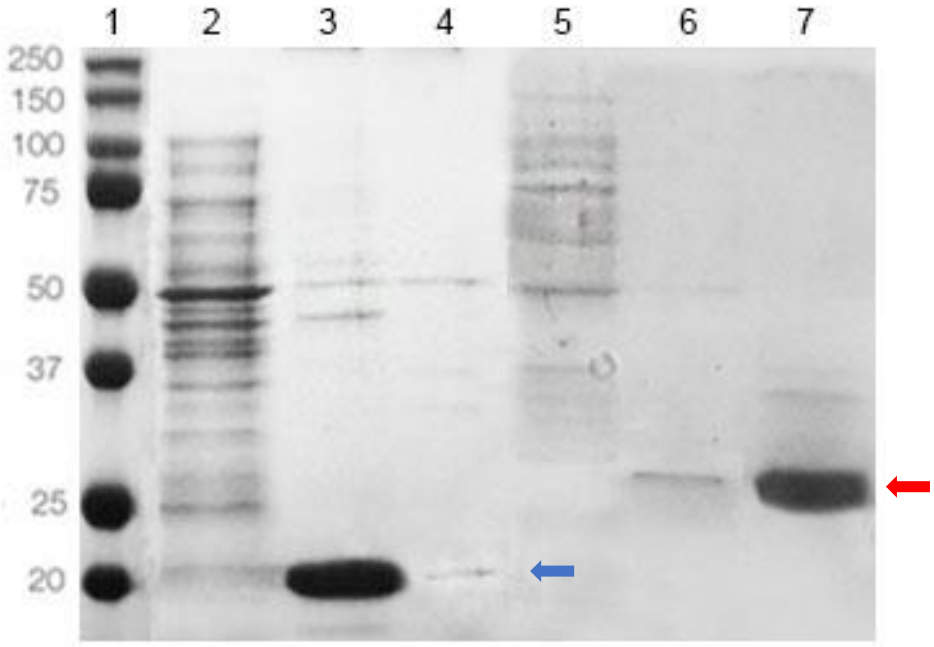

Figure 3.5 SDS-PAGE results of first trial of AS precipitation of EBNA1-HBc (Lane 2-4) and HCV core-HBc (Lane 5-7). Lane 1: molecular weight marker; Lane 2 and 5: supernatant after AS precipitation; Lane 3 and 6: supernatant after resuspension using urea buffer; Lane 4 and 7: pellet after resuspension using urea buffer. (red arrow: HCV core-HBc, blue arrow: EBNA1-HBc)

3.3.2.1 Optimization of acid precipitation of HCV core-HBc

As HCV core-HBc proteins failed to be purified with AS precipitation with a desirable recovery yield, another popular precipitation process used for the purification of recombinant proteins, acid precipitation, was applied. To optimize the acid precipitation condition, six different pH values including pH 7.0, pH 6.5, pH 6.0, pH 5.5, pH 5.0 and pH 4.5 were examined. The precipitated proteins were resuspended

using 4 M urea buffer. The SDS-PAGE result of acid precipitation process for HCV core-HBc is illustrated in **Figure 3.6a**.

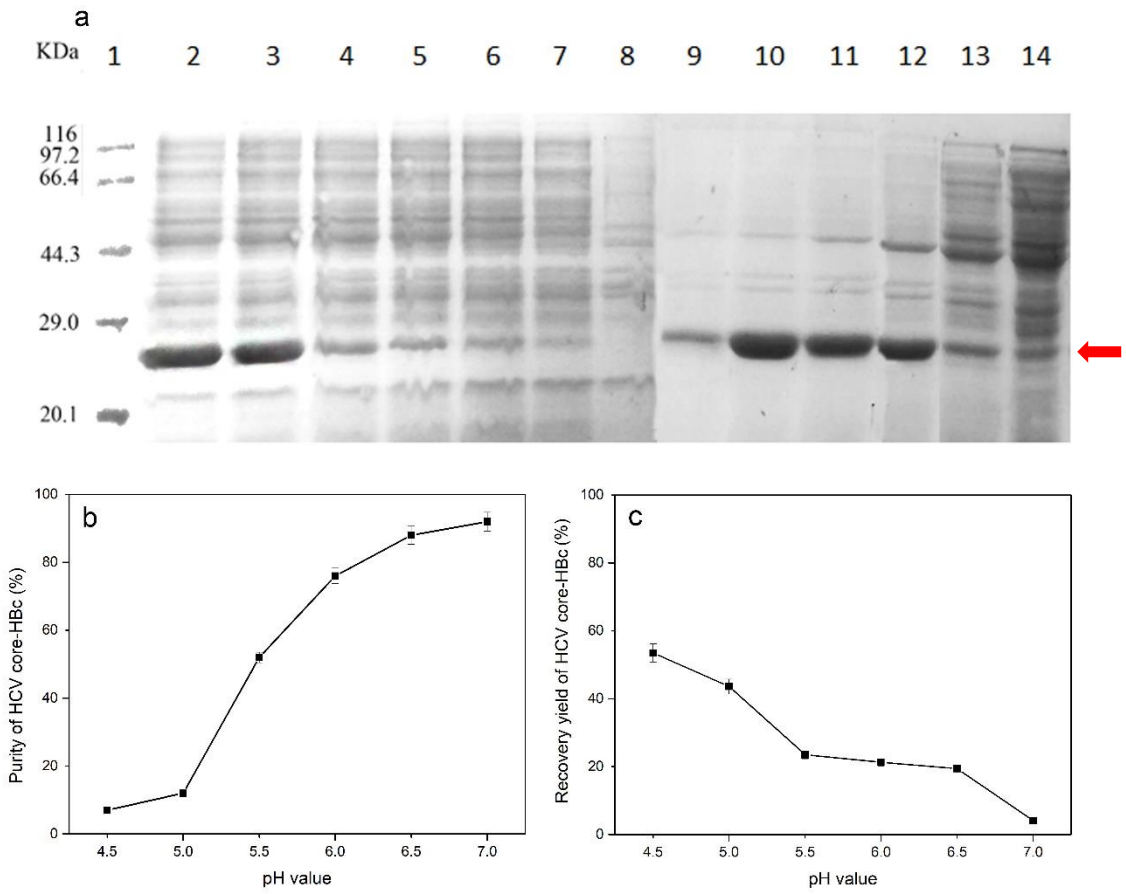

Figure 3.6 Results of acid precipitation of HCV core-HBc. SDS-PAGE images (a), purity (b) and recovery yield (c). SDS-PAGE: Lane 1: molecular weight marker; Lane 2 HCV core-HBc sample before acid precipitation; Lane 3-8: supernatant after precipitation at pH 7.0, 6.5, 6.0, 5.5, 5.0, 4.5; Lane 9-14: supernatant of resuspension after precipitation at pH 7.0, 6.5, 6.0, 5.5, 5.0, 4.5. (red arrow: HCV core-HBc)

In **Figure 3.6b**, HCV core-HBc samples precipitated with pH 7.0 and pH 6.5 achieved high purity of 92 % and 88 % while the purities achieved by other conditions were lower than 80 %. However, the recovery yield of HCV core-HBc sample precipitated at pH 7.0 was only 4.1 % and SDS-PAGE result indicates that there were still HCV core-HBc proteins left in the supernatant of precipitation step. As shown in **Figure 3.6c**, the recovery yield of HCV core-HBc protein after precipitated at pH 6.5 reached 19.4 %.

The achieved recovery yield was found increased with the decrease of pH value (from 21.3 % to 53.5 %), however, the purity of target HCV core-HBc dropped (from 76 % to 7%) (shown in **Figure 3.6b and c**). Therefore, pH 6.5 was considered as the optimal condition for acid precipitation with a high recovery yield and high purity. Even though acid precipitation of HCV core-HBc achieved high purity and good recovery yield, after 24 h storage, the purified HCV core-HBc samples were all aggregated and precipitated.

3.3.2.2 Optimization of AS precipitation of HCV core-HBc

Because of the stability issue observed in the acid precipitation process of HCV core-HBc, optimization of AS precipitation was performed. In the process of AS precipitation of chimeric HBc VLP proteins, there are two major factors, 1) concentration of AS and 2) composition of resuspension buffer that have a great impact on the yield [59]. The optimization of resuspension buffer was firstly performed. Two factors, the pH value for resuspension buffer and concentration of urea , were examined using control variable method. The concentrations of resuspended protein samples were measured, and the recovery yields were calculated for analysis. As shown in **Table 3.2**, comparing different pH value of resuspension buffer, pH 9 buffer obtained the highest recovery yield of 5 % and pH 10 buffer achieved a slightly lower recovery yield of 4.3%. The recovery yields achieved by the other two pH conditions were 2.1 % and 1.1 %. Therefore, pH 9 was chosen as the optimal pH value for the resuspension buffer. Then different urea concentrations were examined. As shown in **Table 3.2**, the recovery yields of HCV core-HBc samples after being resuspended with 4 M urea , 6 M urea and

8 M urea buffers were 5 %, 11.25 % and 26 %, respectively. 8 M urea buffer achieved the highest recovery yield. However, when detecting the resuspended samples with TEM (as shown in **Figure 3.7**), HCV core-HBc sample resuspended with 8 M urea buffer was found to be disassembled. For 6 M urea buffer, HCV core-HBc samples were disassembled partially, while the sample resuspended with 4 M urea buffer remained the fully assembled structure. Therefore, 4 M urea was used as the optimal urea concentration for the resuspension buffer.

Table 3.2 Recovery yields of HCV core-HBc resuspended with different urea buffer.

No.	Concentration of urea	pH	HCV core-HBc yield
1	4 M	10	4.3 %
2	4 M	9	5 %
3	4 M	8	2.1 %
4	4 M	7	1.1 %
5	6 M	9	11.25 %
6	8 M	9	26 %

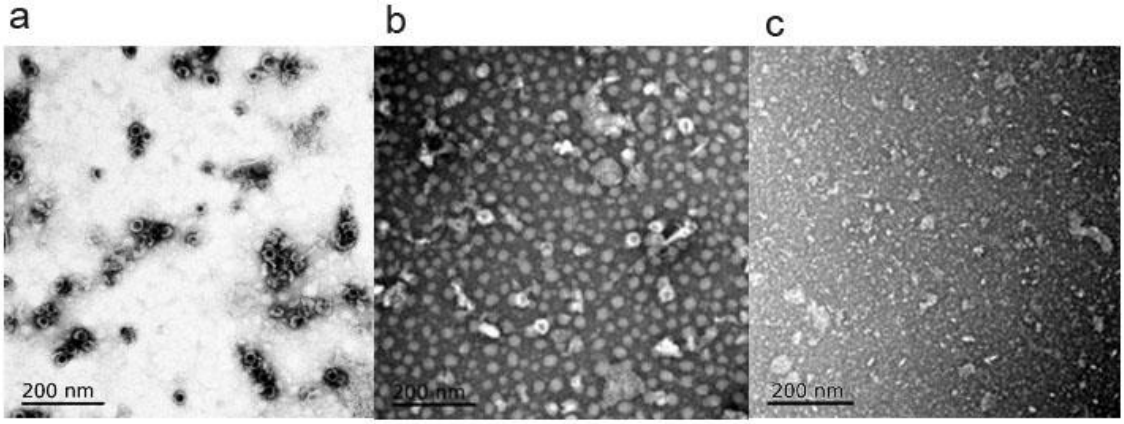

Figure 3.7TEM images of HCV core-HBc sample resuspended using 4 M urea buffer (a), 6 M urea buffer (b) and 8 M urea buffer (c).

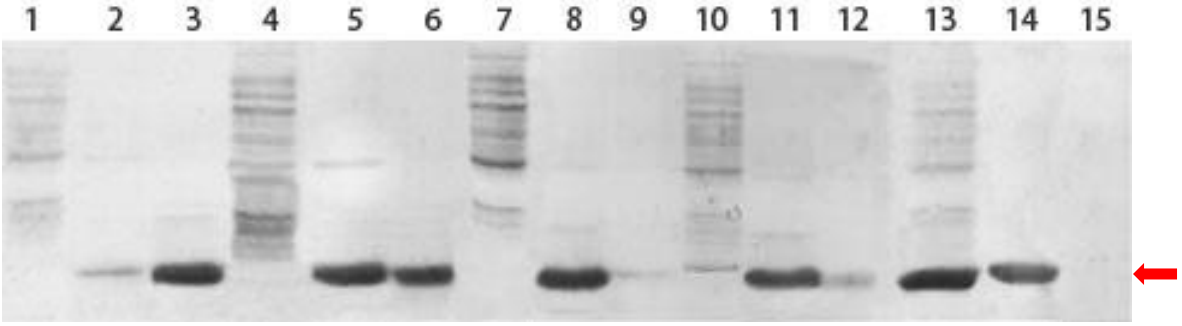

Figure 3.8 SDS-PAGE results of AS precipitation of HCV core-HBc proteins. Lane 1: supernatant after precipitation using 1 M AS; Lane 2: supernatant of resuspension using 4 M urea after precipitation using 1 M AS; Lane 3: pellet of resuspension using 4 M urea buffer after precipitation using 1 M AS; Lane 4: supernatant after precipitation using 0.5 M AS; Lane 5: supernatant of resuspension using 4 M urea after precipitation using 0.5 M AS; Lane 6: pellet of resuspension using 4 M urea buffer after precipitation using 0.5 M AS; Lane 7: supernatant after precipitation using 0.1 M AS; Lane 8: supernatant of resuspension using 4 M urea after precipitation using 0.1 M AS; Lane 9: pellet of resuspension using 4 M urea buffer after precipitation using 0.1 M AS, Lane 10: supernatant after precipitation using 0..05 M AS; Lane 11: supernatant of resuspension using 4 M urea after precipitation using 0.05 M AS; Lane 12: pellet of resuspension using 4 M urea buffer after precipitation using 0.05 M AS, Lane 13: supernatant after precipitation using 0.01 M AS; Lane 14: supernatant of resuspension using 4 M urea after precipitation using 0.01 M AS; Lane 15: pellet of resuspension using 4 M urea buffer after precipitation using 0.01 M AS.(red arrow: HCV core-HBc)

Table 3.3 Recovery yields of HCV core-HBc precipitated with different concentrations of AS

No.	Concentration of AS	Recovery yield of HCV core-HBc
1	1 M	5 %
2	0.5 M	13.5 %
3	0.1 M	22.55 %
4	0.05 M	17.35 %
5	0.01 M	11.2 %

Following the optimization of resuspension buffer, the optimization of AS

concentration was performed. Different concentrations including 0.01 M, 0.05 M, 0.1 M, 0.5 M to 1 M of AS were examined to precipitate the target HCV core-HBc protein from the soluble part of *E. coli* lysate. Then precipitated pellets were resuspended using 4 M urea buffer, pH 9. As shown in **Figure 3.8**, HCV core-HBc protein samples were fully precipitated with 0.1 M AS from the lysate buffer (Lane7-9) and purity of target HCV core-HBc proteins was around 96 % (according to the intensity calculation using SDS-PAGE image). **Table 3.3** demonstrates the recovery yield when using different concentration of AS. Same as the results in SDS-PAGE, 0.1 M AS achieved the best recovery yield with 22.55 %. A slightly lower recovery yield of 17.35 % was obtained when precipitating with 0.05 M AS, while the recovery yield achieved by other concentrations were relatively low. In addition, it was found that with higher concentrations of AS (1 M and 0.5 M) target HCV core-HBc proteins were able to fully precipitate. However, these precipitated protein samples cannot be resuspended with 4 M urea buffer with high yield (Lane 1-6). In contrast, lower concentration of AS had a greater loss of target proteins in the precipitation step, but the resuspension of target proteins was efficient (Lane 10-15). After optimization, the resuspended HCV core-HBc proteins was examined together with EBNA1-HBc proteins using TEM. **Figure 3.9** proves that the purified HCV core-HBc and EBNA1-HBc proteins were in fully assembled VLP structure.

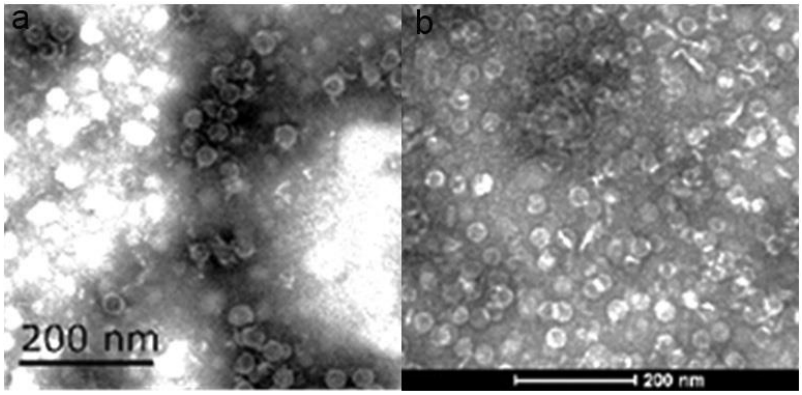

Figure 3.9 TEM images of supernatant of HCV core-HBc after 0.1 M AS precipitation (a) and EBNA1-HBc (b) after 1 M AS precipitation.

3.3.3 Nucleic acid impurities removal by ion exchange chromatography

To further remove nucleic acid impurities from produced chimeric HBc VLP proteins, HCV core-HBc was chosen as the target. As theoretical pI for HCV core-HBc is 11.03, binding strategy using two cationic exchange chromatgraphy, HiTrap CM Sepharose FF and HiTrap SP Sepharose FF chromatography, were applied after resuspension of HCV core-HBc proteins with 4 M urea buffer, pH 9. As shown in **Figure 3.10**, HCV core-HBc samples failed to bind with both of two cationic exchange columns. All HCV core-HBc samples were in the flowthrough (FT) fraction. The protein concentrations in FT fraction were similar to the concentration of the sample loaded (shown in **Table 3.4**).

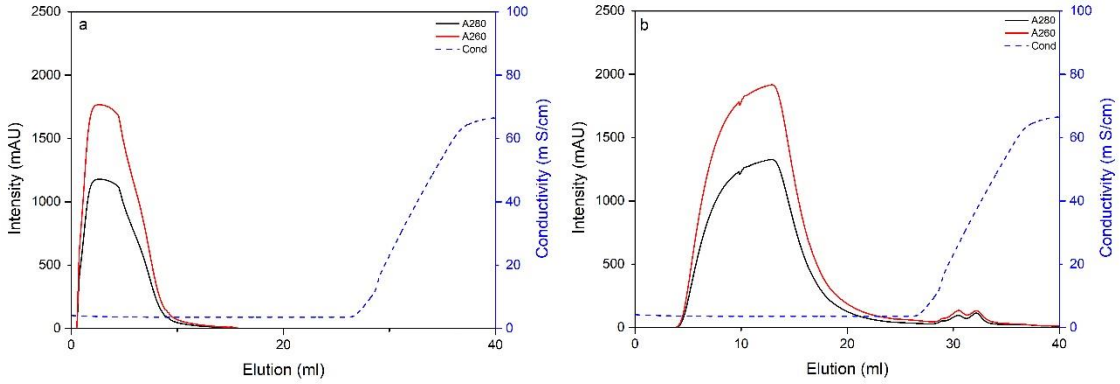

Figure 3.10 Chromatograms of HCV core-HBc protein using HiTrap CM Sepharose FF (a) and HiTrap SP Sepharose FF (b) chromatography.

Table 3.4 Concentration of protein in different fractions.

No.	Fraction	Concentration of protein (mg/ml)
1	Before loading to chromatography	2.08
2	FT fraction of CM chromatography	1.91
3	FT fraction of SP chromatography	1.87

Then, a flowthrough strategy was applied using three anion exchange chromatography (AEC) resins including Capto™ Q chromatography, HiTrap® Q FF chromatography and POROS™ 50 HQ chromatography. **Figure 3.11** illustrates the chromatography results of HCV core-HBc VLP purified with Capto™ Q chromatography, HiTrap® Q FF chromatography and POROS™ 50 HQ chromatography. Two fractions, FT fraction and elution fraction, were clearly observed. **Figure 3.12a and b** demonstrates the SDS-PAGE and DNA gel electrophoresis results of different fractions in the chromatography. More than 95 % of HCV core-HBc proteins remained in the FT fraction of all three columns. However, as indicated by DNA gel electrophoresis results, around 80 % and

40 % of nucleic acids remained in the FT fraction of Capto™ Q and HiTrap® Q FF chromatography, respectively. In contrast, POROS™ 50 HQ chromatography obtained a good ability to remove nucleic acids from HCV core-HBc proteins. Less than 10 % of nucleic acids left in the FT fraction of POROS™ 50 HQ chromatography, and 90 % of nucleic acids were in the elution fraction. Therefore, POROS™ 50 HQ chromatography is potentially more suitable to remove nucleic acid impurities for HCV core-HBc proteins. Afterwards, HCV core-HBc sample was dialysed against 20 mM PB, pH 7.4 to eliminate urea to be prepared for the following *in vivo* immunogenicity experiments. TEM image of dialysed HCV core-HBc proteins was taken and VLP structures of HCV core-HBc were clearly observed (shown in **Figure 3.12c**). However, after being stored for a short period, irrevesible precipitation of all purified HCV core-HBc samples were found.

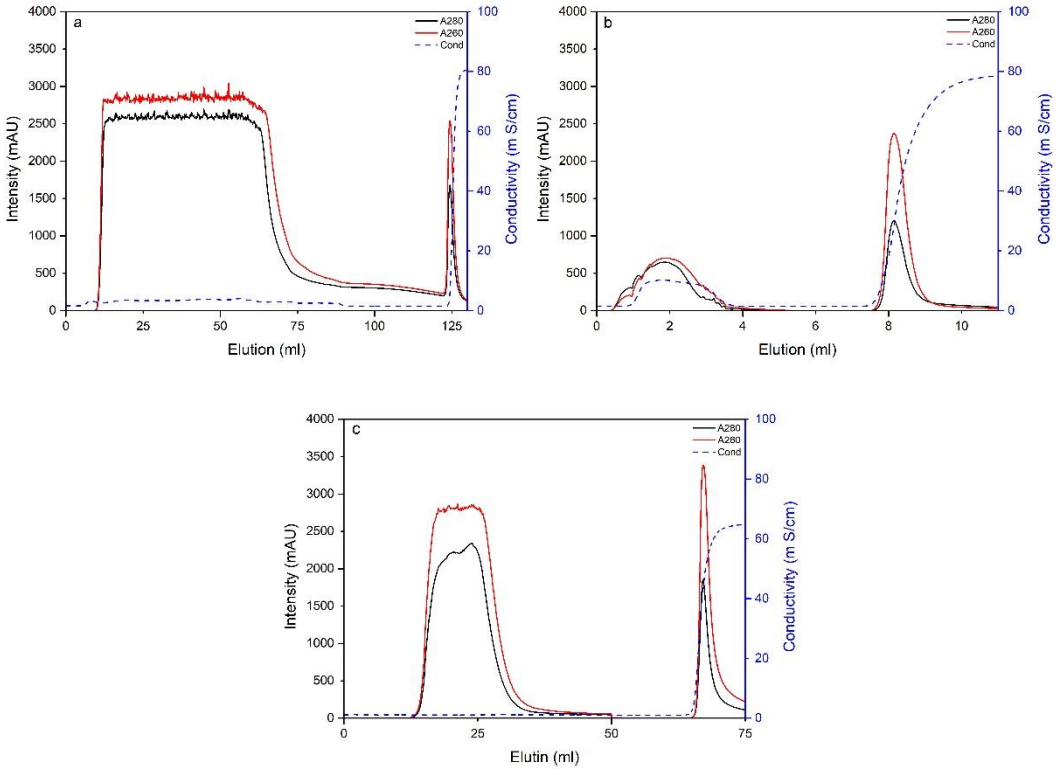

Figure 3.11 Purification of HCV core-HBc by Capto™ Q chromatography (a), HiTrap® Q FF chromatography (b) and POROS™ 50 HQ chromatography (c) using 4 M urea buffer, pH 9 as equilibration buffer and 4 M urea buffer with 100 mM sodium sulphate, pH 9 as elution buffer.

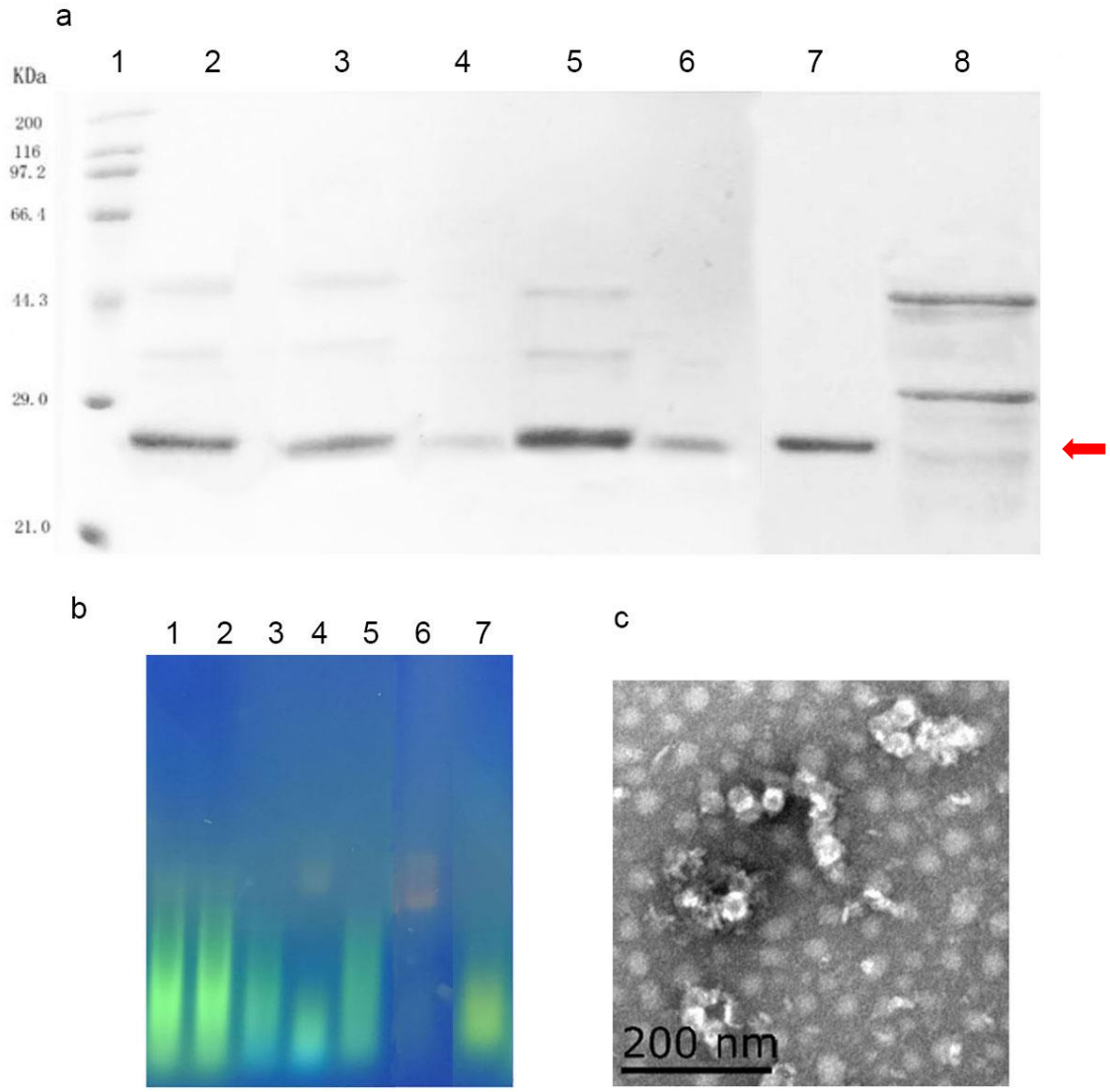

Figure 3.12 SDS-PAGE (a) and DNA gel electrophoresis (b) of HCV core-HBc samples in Capto™ Q, HiTrap® Q FF and POROS™ 50 HQ chromatography and TEM image (c) of FT fraction from POROS™ 50 HQ chromatography. SDS-PAGE: Lane 1: molecular weight marker; Lane 2: HCV core-HBc sample before loading to chromatography; Lane 3: FT fraction of Capto™ Q chromatography; Lane 4: Elution fraction of Capto™ Q chromatography; Lane 5: FT fraction of HiTrap® Q FF chromatography; Lane 6: Elution fraction of HiTrap® Q FF chromatography, Lane 7: FT fraction of POROS™ 50 HQ chromatography; Lane 8: Elution fraction of POROS™ 50 HQ chromatography. DNA gel: Lane 1: HCV core-HBc sample before loading to chromatography; Lane 2: FT fraction of Capto™ Q chromatography; Lane 3: Elution fraction of Capto™ Q chromatography; Lane 4: FT fraction of HiTrap® Q FF chromatography; Lane 5: Elution fraction of HiTrap® Q FF chromatography, Lane 6: FT fraction of POROS™ 50 HQ chromatography; Lane 7: Elution fraction of POROS™ 50 HQ chromatography. (red arrow: HCV core-HBc)

3.4 Discussion

In the development of vaccines for oncoviruses, virus like particles (VLPs) have shown great potentials to induce strong and long-term immune responses due to their unique structure[44]. To target different infection origins, recombinant protein technique has been applied by fusing target antigens to selected VLP carriers[45]. Hepatitis B core VLP, one of the broadly studied VLP candidates, demonstrates a good ability to display foreign epitopes on the surface of its VLP structure and have been reported to elicit strong *in vivo* epitope specific immunogenicity[44]. However, in the production of chimeric HBc VLP vaccines targeting different infection origins, challenges in expression and purification remain[20, 46].

The primary objectives of this chapter are 1) expression of the chimeric HBc VLP proteins presenting HCV core epitope and EBNA1 epitope in soluble form using *E. coli* expression system, 2) optimization of the purification process of these two chimeric VLP proteins to achieve high production yields and 3) removal of nuclear acid impurities from chimeric HBc VLP proteins.

Both HCV core epitope and EBNA1 epitope were fused to the N-terminal of HBc VLP by gene engineering to expose them on the surface of HBc VLPs. HCV core-HBc plasmid was successfully constructed using overlapped PCR and EBNA1-HBc VLP was supplied by Beijing Genomics Institute, China. In the production of chimeric VLP-based vaccines, *E. coli* has been chosen as an efficient host cell to express VLP proteins for high production yield[47]. The expression of HCV core-HBc and EBNA1-HBc

proteins were followed the conditions for the expression of wild type (wt) HBc as described in previous work in IPE[38]. Around 97 % of EBNA1-HBc was successfully expressed in the soluble form with the expression condition for wt HBc while only around 20 % HCV core-HBc proteins were expressed in soluble form. This could be because of the insertion of different epitopes as EBNA1 is a 11-mer and non-structural epitope while HCV core is a 44-mer epitope (4 times larger than EBNA1) with α-helix which potentially increases the difficulty for *E. coli* to fold the proteins into the correct structure. Therefore, a control variable of the expression conditions including cell density for induction, expression temperature and concentration of IPTG was conducted. After optimization, 95 % of target HCV core-HBc proteins were successfully expressed in soluble form in *E. coli* with cell density for induction at OD_{600} 0.8, expression temperature at 37 ℃ and 0.5 mM IPTG. In addition, it was found that expression temperature could have a minor impact on the improvement of soluble expression for chimeric HBc proteins while the other two factors which could directly influence the expression rate of chimeric HBc proteins have larger influence of soluble expression of chimeric HBc proteins.

After successfully expression of HCV core-HBc and EBNA1-HBc in soluble form using *E. coli*, both chimeric HBc proteins were purified using Ammonium Sulphate (AS) precipitation following the process described in previous work in IPE[38]. In the process, 1 M AS was applied for the precipitation and 4 M urea buffer, pH 9 was used as resuspension buffer. The result indicates that EBNA1-HBc proteins could be purified

with recovery yield of 39.1 % and the purity reached 95 % while the recovery yield of HCV core-HBc was only 5 %. Most of the target HCV core-HBc proteins remained in the pellets after resuspension. Chen et al. (2015) found a similar issue and they claimed that high concentration of AS potentially caused the change in interaction between subunits in the VLPs and form strong aggregation[48]. Therefore, two plans were formed. For the first plan. acid precipitation, one popular purification approach for recombinant proteins, was applied and optimized to replace the AS precipitation. The acid precipitation result indicates that pH 6.5 was the optimal condition to obtain the high recovery yield of 19.4 % and high purity of 88 % for target HCV core-HBc proteins. The achieved recovery yield and purity are lower compared with EBNA1-HBc sample purified using AS precipitation. In addition, HCV core-HBc sample purified with acid precipitation at pH 6.5 was found instable and the sample aggregated and precipitated after storage for 24 hours. This suggests that acid precipitation is potentially not suitable for the purification of HCV core-HBc proteins.

For the second plan, AS precipitation process for HCV core-HBc proteins was optimized. Two factors including resuspension buffer and concentration of AS in AS precipitation process was optimized to achieve high recovery yield of HCV core-HBc with high purity. As a result, 4 M urea buffer, pH 9 and 0.1 M AS were determined as the optimal conditions for AS precipitation for HCV core-HBc. With the optimized condition, the recovery yield of HCV core-HBc reached 22.5 % with a purity of 96 %. Purified EBNA1-HBc and HCV core-HBc proteins were then dialysed against 20 mM

PB, pH 7.4 for the evaluation of production yield. The production yields of chimeric HCV core-HBc VLP and EBNA1-HBc VLP were 161.6 mg/L or 40.4 mg/g of wet cell weight (HCV core-HBc) and 248.4 mg/L or 62.1 mg/g of wet cell weight (EBNA1-HBc), respectively. The achieved yields of our chimeric HBc VLPs are significantly higher than other reported HBc VLPs of 6.4 mg/L produced using Cell-free protein synthesis (CFPS) system[49] and 3.21 mg/L produced using *E. coli* expression system in inclusion bodies[50]. In addition, our achieved yields are also higher than the reportedly high production yield in *P. pastoris* of 3 mg/g of wet cell weight[51]. In addition to high production yield, TEM images prove that purified HCV core-HBc and EBNA1-HBc proteins were in assembled VLP structure.

After being purified with AS precipitation, chimeric HCV core-HBc proteins were loaded to ion exchange chromatography for the preliminary study to eliminate the nucleic acid impurities from chimeric VLP samples. Binding strategy with cationic exchange chromatography, HiTrap CM Sepharose FF and HiTrap SP Sepharose FF chromatography was designed to have HCV core-HBc proteins bind with column while remain nuclear acid in the FT fraction according to the pI information of HCV core-HBc. However, it was found that HCV core-HBc proteins failed to bind with column and all samples were in the FT fraction together with nucleic acid impurities. Therefore, a flowthrough strategy was designed and three types of anionic exchange chromatography including Capto™ Q[52, 53], HiTrap® Q FF[54, 55] and POROS™ 50 HQ[56-58] chromatography that have been reported to be applied in the removal of

nucleic acids from chimeric VLP proteins were employed. DNA gel electrophoresis results indicate that both Capto™ Q and HiTrap® Q FF resins are not suitable for the removal of nucleic acid impurities for HCV core-HBc VLP as 80 % and 40 % of nucleic acids remained in the FT fractions of two chromatography, respectively. For POROS™ 50 HQ chromatography, 90 % of nucleic acid impurities were bound with the column and observed in the elution fraction, and 95 % of target HCV core-HBc VLP samples remained in the flowthrough fraction. TEM image also confirms the assembled VLP structure of HCV core-HBc after removal of nucleic acid impurities. However, all HCV core-HBc proteins after removal of nucleic acids were found to aggregate and precipitate after a short period storage. This could result from the instability of HCV core-HBc subunit caused by the absence of nucleic acids, because HBc protein has the arginine-rich domain (ARD) at the C terminal. Le Pogam' group and Chua's group have made hypothesis that missing the nucleic acids can lead to the instability of the HBc VLP structure due to the charge balance and chaperone activity[59, 60].

3.5 Conclusion

In this project, gene sequences of HCV core epitope and EBNA1 epitope targeting HCV and EBV respectively were successfully fused with HBc VLP sequence at 5'-terminal in pET 30a plasmid. pET 30a-HCV core-HBc and pET 30a-EBNA1-HBc were transferred into *E. coli* BL21 (DE) for expression. Both HCV core-HBc and EBNA1-HBc proteins were successfully expressed in the soluble form with high production yields which are 40.4 mg/g and 62.1 mg/g of wet cell weight, respectively. It was found

that control of expression rate is essential for expression of chimeric HBc protein in soluble form. Both HCV core-HBc and EBNA1-HBc protein were found to be expressed as VLP structures in *E. coli*. In addition, it was found that the nature of presented antigens of chimeric HBc proteins can influence the expression of chimeric HBc protein in soluble form as long and structural epitope (HCV core) has a larger impact than short and non-structured epitope (EBNA1). The optimization of AS precipitation process of expressed VLP protein samples were then performed. Results indicate that 0.1 M AS was the optimal AS concentration for HCV core-HBc. After optimization, the recovery yield of HCV core-HBc improved from 5 % to 22.5 % with a purity of 96 %. On the other hand, 1 M AS was the optimal AS concentration for EBNA1-HBc precipitation. The precipitated chimeric HBc VLP samples were then resuspended with 4 M urea buffer, pH 9. It was also found that the insertion of a longer and structural epitope, HCV core-HBc, can change the property of chimeric HBc VLP in AS precipitation and irreversible precipitation can be formed with high concentration of AS. Finally, POROS™ 50 HQ chromatography is proven to remove nucleic acid impurities from HCV core-HBc proteins effectively. However, HCV core-HBc proteins after removal of nucleic acids were found to be precipitated after short period storage due to the charge balance hypothesis and chaperone activity. Further study on the stability and structure of the chimeric VLP presenting different epitopes is described in next chapter.

References

[1] H.K. Ong, W.S. Tan, K.L. Ho, Virus like particles as a platform for cancer vaccine development, PeerJ 5 (2017) 4053-4053.

[2] S. Buffin, I. Peubez, F. Barrière, M.-C. Nicolaï, T. Tapia, V. Dhir, E. Forma, N. Sève, I. Legastelois, Influenza A and B virus-like particles produced in mammalian cells are highly immunogenic and induce functional antibodies, Vaccine 37(46) (2019) 6857-6867.

[3] A. Kazaks, I.N. Lu, S. Farinelle, A. Ramirez, V. Crescente, B. Blaha, O. Ogonah, T. Mukhopadhyay, M.P. de Obanos, A. Krimer, I. Akopjana, J. Bogans, V. Ose, A. Kirsteina, T. Kazaka, N.J. Stonehouse, D.J. Rowlands, C.P. Muller, K. Tars, W.M. Rosenberg, Production and purification of chimeric HBc virus-like particles carrying influenza virus LAH domain as vaccine candidates, BMC Biotechnol. 17(1) (2017) 79.

[4] S. Kruger, M. Ilmer, S. Kobold, B.L. Cadilha, S. Endres, S. Ormanns, G. Schuebbe, B.W. Renz, J.G. D'Haese, H. Schloesser, V. Heinemann, M. Subklewe, S. Boeck, J. Werner, M. von Bergwelt-Baildon, Advances in cancer immunotherapy 2019 – latest trends, J. Exp. Clin. Cancer Res. 38(1) (2019) 268.

[5] P.J. Farrell, Epstein–Barr Virus and Cancer, Annual Review of Pathology: Mechanisms of Disease 14(1) (2019) 29-53.

[6] A.F. Sander, P.L. Lollini, Virus-like antigen display for cancer vaccine development, what is the potential?, Expert Rev Vaccines 17(4) (2018) 285-288.

[7] P. Pumpens, E. Grens, HBV core particles as a carrier for B cell/T cell epitopes, Intervirology 44(2-3) (2001) 98-114.

[8] K. Roose, S.D. Baets, B. Schepens, X. Saelens, Hepatitis B core–based virus–like particles to present heterologous epitopes, Expert review of vaccines 12(2) (2013) 183-198.

[9] I.F.M. Suffian, J.T.W. Wang, F.N. Faruqu, J. Benitez, Y. Nishimura, C. Ogino, A. Kondo, K.T. Al-Jamal, Engineering Human Epidermal Growth Receptor 2-Targeting Hepatitis B Virus Core Nanoparticles for siRNA Delivery in Vitro and in Vivo, ACS Applied Nano Materials 1(7) (2018) 3269-3282.

[10] I.F.B. Mohamed Suffian, J.T.-W. Wang, N.O. Hodgins, R. Klippstein, M. Garcia-Maya, P. Brown, Y. Nishimura, H. Heidari, S. Bals, J.K. Sosabowski, C. Ogino, A. Kondo, K.T. Al-Jamal, Engineering hepatitis B virus core particles for targeting HER2 receptors in vitro and in vivo, Biomaterials 120 (2017) 126-138.

[11] A. Strods, V. Ose, J. Bogans, I. Cielens, G. Kalnins, I. Radovica, A. Kazaks, P. Pumpens, R. Renhofa, Preparation by alkaline treatment and detailed characterisation of empty hepatitis B virus core particles for vaccine and gene therapy applications, Sci. Rep. 5(1) (2015) 11639.

[12] Y. Zhang, S. Song, C. Liu, Y. Wang, X. Xian, Y. He, J. Wang, F. Liu, S. Sun, Generation of chimeric HBc proteins with epitopes in E.coli: Formation of virus-like particles and a potent inducer of antigen-specific cytotoxic immune response and anti-tumor effect in vivo, Cellular Immunology 247(1) (2007) 18-27.

[13] Y. Lei, J. Shao, F. Zhao, Y. Li, C. Lei, F. Ma, H. Chang, Y. Zhang, Artificially designed hepatitis B virus core particles composed of multiple epitopes of type A and O foot-and-

mouth disease virus as a bivalent vaccine candidate, J. Med. Virol. 91(12) (2019) 2142-2152.
[14] X. Ye, Z. Ku, Q. Liu, X. Wang, J. Shi, Y. Zhang, L. Kong, Y. Cong, Z. Huang, Chimeric Virus-Like Particle Vaccines Displaying Conserved Enterovirus 71 Epitopes Elicit Protective Neutralizing Antibodies in Mice through Divergent Mechanisms, J. Virol. 88(1) (2014) 72.
[15] D.S. Peabody, B. Manifold-Wheeler, A. Medford, S.K. Jordan, J. do Carmo Caldeira, B. Chackerian, Immunogenic display of diverse peptides on virus-like particles of RNA phage MS2, J Mol Biol 380(1) (2008) 252-63.
[16] J.C. Caldeira, D.S. Peabody, Thermal stability of RNA phage virus-like particles displaying foreign peptides, J Nanobiotechnology 9 (2011) 22.
[17] J. Schumacher, T. Bacic, R. Staritzbichler, M. Daneschdar, T. Klamp, P. Arnold, S. Jagle, O. Tureci, J. Markl, U. Sahin, Enhanced stability of a chimeric hepatitis B core antigen virus-like-particle (HBcAg-VLP) by a C-terminal linker-hexahistidine-peptide, J Nanobiotechnology 16(1) (2018) 39.
[18] M.T. Arevalo, T.M. Wong, T.M. Ross, Expression and Purification of Virus-like Particles for Vaccination, Journal of visualized experiments : JoVE (112) (2016) 54041.
[19] M. Merlin, E. Gecchele, S. Capaldi, M. Pezzotti, L. Avesani, Comparative Evaluation of Recombinant Protein Production in Different Biofactories: The Green Perspective, BioMed Research International 2014 (2014) 136419.
[20] G.L. Rosano, E.A. Ceccarelli, Recombinant protein expression in Escherichia coli: advances and challenges, Front. Microbiol. 5(172) (2014).
[21] X. Huang, X. Wang, J. Zhang, N. Xia, Q. Zhao, Escherichia coli-derived virus-like particles in vaccine development, NPJ vaccines 2 (2017) 3.
[22] A. Zeltins, Construction and Characterization of Virus-Like Particles: A Review, Mol. Biotechnol. 53(1) (2013) 92-107.
[23] C. Ladd Effio, P. Baumann, C. Weigel, P. Vormittag, A. Middelberg, J. Hubbuch, High-throughput process development of an alternative platform for the production of virus-like particles in Escherichia coli, J Biotechnol 219 (2016) 7-19.
[24] W. Dai, P. Xiong, X. Zhang, Z. Liu, J. Chen, Y. Zhou, X. Ye, C. Zhang, Recombinant virus-like particle presenting a newly identified coxsackievirus A10 neutralization epitope induces protective immunity in mice, Antiviral Res. 164 (2019) 139-146.
[25] H.P. Sørensen, K.K. Mortensen, Soluble expression of recombinant proteins in the cytoplasm of Escherichia coli, Microbial Cell Factories 4(1) (2005) 1.
[26] A.M. Al-Hejin, R.S. Bora, M.M.M. Ahmed, Plasmids for Optimizing Expression of Recombinant Proteins in E. coli, Plasmid, IntechOpen2019.
[27] I. Rabhi-Essafi, A. Sadok, N. Khalaf, D.M. Fathallah, A strategy for high-level expression of soluble and functional human interferon α as a GST-fusion protein in E.coli, Protein Engineering, Design and Selection 20(5) (2007) 201-209.
[28] J. Li, Q. Han, T. Zhang, J. Du, Q. Sun, Y. Pang, Expression of soluble native protein in Escherichia coli using a cold-shock SUMO tag-fused expression vector, Biotechnology Reports 19 (2018) e00261.

[29] K.M. Frietze, D.S. Peabody, B. Chackerian, Engineering virus-like particles as vaccine platforms, Curr. Opin. Virol. 18 (2016) 44-49.

[30] N.K.C. Linda H.L. Lua, Frank Sainsbury, Yap P. Chuan,Nani Wibowo, Anton P.J. Middelberg, Bioengineering Virus-Like Particles as Vaccines, Biotechnology and Bioengineering 111 (2014) 425-440.

[31] J. Freivalds, A. Dislers, V. Ose, P. Pumpens, K. Tars, A. Kazaks, Highly efficient production of phosphorylated hepatitis B core particles in yeast Pichia pastoris, Protein Expr Purif 75(2) (2011) 218-24.

[32] M. Zahin, J. Joh, S. Khanal, A. Husk, H. Mason, H. Warzecha, S.-j. Ghim, D.M. Miller, N. Matoba, A.B. Jenson, Scalable Production of HPV16 L1 Protein and VLPs from Tobacco Leaves, PLoS One 11(8) (2016) 160995.

[33] A. Kazaks, I.N. Lu, S. Farinelle, A. Ramirez, V. Crescente, B. Blaha, O. Ogonah, T. Mukhopadhyay, M.P. de Obanos, A. Krimer, I. Akopjana, J. Bogans, V. Ose, A. Kirsteina, T. Kazaka, N.J. Stonehouse, D.J. Rowlands, C.P. Muller, K. Tars, W.M. Rosenberg, Production and purification of chimeric HBc virus-like particles carrying influenza virus LAH domain as vaccine candidates, BMC biotechnology 17(1) (2017) 79-79.

[34] D. Rajalingam, C. Loftis, J.J. Xu, T.K.S. Kumar, Trichloroacetic acid-induced protein precipitation involves the reversible association of a stable partially structured intermediate, Protein science : a publication of the Protein Society 18(5) (2009) 980-993.

[35] P. Pereira Aguilar, T.A. Schneider, V. Wetter, D. Maresch, W.L. Ling, A. Tover, P. Steppert, A. Jungbauer, Polymer-grafted chromatography media for the purification of enveloped virus-like particles, exemplified with HIV-1 gag VLP, Vaccine 37(47) (2019) 7070-7080.

[36] C. Ladd Effio, S.A. Oelmeier, J. Hubbuch, High-throughput characterization of virus-like particles by interlaced size-exclusion chromatography, Vaccine 34(10) (2016) 1259-67.

[37] Y. Yang, Y. Mengran, S. Zhang, G. Ma, Z. Su, Adsorption of virus-like particles on ion exchange surface: Conformational changes at different pH detected by dual polarization interferometry, Journal of Chromatography A 1408 (2015) 161-168.

[38] Y. Zhang, Y. Liu, B. Zhang, S. Yin, X. Li, D. Zhao, W. Wang, J. Bi, Z. Su, In vitro preparation of uniform and nucleic acid free hepatitis B core particles through an optimized disassembly-purification-reassembly process, Protein Expression and Purification (2020) 105747.

[39] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry 72(1) (1976) 248-254.

[40] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, Nat. Methods 9(7) (2012) 671-675.

[41] E.A. Blokhina, V.V. Kuprianov, L.A. Stepanova, L.M. Tsybalova, O.I. Kiselev, N.V. Ravin, K.G. Skryabin, A molecular assembly system for presentation of antigens on the surface of HBc virus-like particles, Virology 435(2) (2013) 293-300.

[42] A.V. Bryksin, I. Matsumura, Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids, BioTechniques 48(6) (2010) 463-465.

[43] Y. Zhang, S. Yin, B. Zhang, J. Bi, Y. Liu, Z. Su, HBc-based virus-like particle assembly from inclusion bodies using 2-methyl-2, 4-pentanediol, Process Biochem. (2019).

[44] K. Roose, S. De Baets, B. Schepens, X. Saelens, Hepatitis B core-based virus-like particles to present heterologous epitopes, Expert Rev Vaccines 12(2) (2013) 183-98.

[45] S.L. Stephen, L. Beales, H. Peyret, A. Roe, N.J. Stonehouse, D.J. Rowlands, Recombinant Expression of Tandem-HBc Virus-Like Particles (VLPs), Methods Mol Biol 1776 (2018) 97-123.

[46] J. Fuenmayor, F. Gòdia, L. Cervera, Production of virus-like particles for vaccines, N. Biotechnol. 39(Pt B) (2017) 174-180.

[47] Y. Xiao, H.Y. Chen, Y. Wang, B. Yin, C. Lv, X. Mo, H. Yan, Y. Xuan, Y. Huang, W. Pang, X. Li, Y.A. Yuan, K. Tian, Large-scale production of foot-and-mouth disease virus (serotype Asia1) VLP vaccine in Escherichia coli and protection potency evaluation in cattle, BMC Biotechnol. 16(1) (2016) 56.

[48] Y. Chen, Y. Zhang, C. Quan, J. Luo, Y. Yang, M. Yu, Y. Kong, G. Ma, Z. Su, Aggregation and antigenicity of virus like particle in salt solution--A case study with hepatitis B surface antigen, Vaccine 33(35) (2015) 4300-6.

[49] A.J. Spice, R. Aw, D.G. Bracewell, K.M. Polizzi, Synthesis and Assembly of Hepatitis B Virus-Like Particles in a Pichia pastoris Cell-Free System, Frontiers in Bioengineering and Biotechnology 8(72) (2020).

[50] I.F. Bin Mohamed Suffian, M. Garcia-Maya, P. Brown, T. Bui, Y. Nishimura, A.R.B.M.J. Palermo, C. Ogino, A. Kondo, K.T. Al-Jamal, Yield Optimisation of Hepatitis B Virus Core Particles in E. coli Expression System for Drug Delivery Applications, Sci. Rep. 7(1) (2017) 43160.

[51] J. Freivalds, A. Dislers, V. Ose, P. Pumpens, K. Tars, A. Kazaks, Highly efficient production of phosphorylated hepatitis B core particles in yeast Pichia pastoris, Protein Expression and Purification 75(2) (2011) 218-224.

[52] T. Weigel, T. Solomaier, A. Peuker, T. Pathapati, M.W. Wolff, U. Reichl, A flow-through chromatography process for influenza A and B virus purification, J. Virol. Methods 207 (2014) 45-53.

[53] S. Chandramouli, A. Medina-Selby, D. Coit, M. Schaefer, T. Spencer, L.A. Brito, P. Zhang, G. Otten, C.W. Mandl, P.W. Mason, Generation of a parvovirus B19 vaccine candidate, Vaccine 31(37) (2013) 3872-3878.

[54] T.A.d.C. Lamounier, L.M.d. Oliveira, B.R.d. Camargo, K.B. Rodrigues, E.F. Noronha, B.M. Ribeiro, T. Nagata, Production of Brazilian human norovirus VLPs and comparison of purification methods, Brazilian Journal of Microbiology 46(4) (2015) 1265-1268.

[55] H.W. Ng, M.F.X. Lee, G.K. Chua, B.K. Gan, W.S. Tan, C.W. Ooi, S.Y. Tang, E.S. Chan, B.T. Tey, Size-selective purification of hepatitis B virus-like particle in flow-through chromatography: Types of ion exchange adsorbent and grafted polymer architecture, J. Sep. Sci. 41(10) (2018) 2119-2129.

[56] K. Stubenrauch, A. Bachmann, R. Rudolph, H. Lilie, Purification of a viral coat protein by an engineered polyionic sequence, Journal of Chromatography B: Biomedical Sciences and Applications 737(1-2) (2000) 77-84.

[57] N. Landry, B.J. Ward, S. Trépanier, E. Montomoli, M. Dargis, G. Lapini, L.-P. Vézina, Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza, PLoS One 5(12) (2010) e15559.

[58] N. Landry, S. Pillet, D. Favre, J.-F. Poulin, S. Trépanier, B. Yassine-Diab, B.J. Ward, Influenza virus-like particle vaccines made in Nicotiana benthamiana elicit durable, poly-functional and cross-reactive T cell responses to influenza HA antigens, Clin. Immunol. 154(2) (2014) 164-177.

[59] S. Le Pogam, P.K. Chua, M. Newman, C. Shih, Exposure of RNA templates and encapsidation of spliced viral RNA are influenced by the arginine-rich domain of human hepatitis B virus core antigen (HBcAg 165-173), J. Virol. 79(3) (2005) 1871-1887.

[60] P.K. Chua, F.-M. Tang, J.-Y. Huang, C.-S. Suen, C. Shih, Testing the Balanced Electrostatic Interaction Hypothesis of Hepatitis B Virus DNA Synthesis by Using an In Vivo Charge Rebalance Approach, J. Virol. 84(5) (2010) 2340-2351.

Chapter 4 Characterization and stability evaluation of chimeric HBc VLPs presenting foreign epitopes aiding with computational protein modelling

Abstract

In the development of chimeric virus like particle (VLP) based cancer vaccines, the stability of produced vaccines is essential to ensure its potency to induce strong antigen specific immune response. In this chapter, the study aims to investigate and evaluate the influences of insertion of foreign epitopes on the stability of chimeric HBc VLPs after insertion of foreign epitopes assisted with computational protein modelling. Two different epitopes, short and non-structural Epstein-Barr nuclear antigen 1 (EBNA1) (aa: 407-417) and long and structural Hepatitis C virus core (HCV core) (aa: 10-53) were fused with HBc VLP to form chimeric HBc VLPs. Chimeric EBNA1-HBc and HCV core-HBc were expressed and purified using *Escherichia coli* (*E. coli*) under optimized conditions achieved in **Chapter 3**. Produced chimeric HBc proteins showed similar morphology and correctly assembled VLP structure of chimeric HBc proteins were observed after purification. The evaluation of the stability of chimeric HBc VLPs under different physical (freeze/thaw cycles and temperature) and chemical stresses (Sodium dodecyl sulphate (SDS) and pH) is performed in comparison with wild type

(wt) HBc VLP. HCV core-HBc VLP was found to form irreversible precipitates during the evaluation and was sensitive to the applied stresses. In contrast, EBNA1-HBc VLP showed similar stabilities to wt HBc VLP under all tested physical stresses, however, its stability was found to be lower when treating with 0.06 % SDS or in the environment at pH 5 compared with wt HBc VLP. To investigate the mechanism behind these findings, 3D protein structures of both chimeric HCV core-HBc and EBNA1-HBc monomer were investigated using computational protein modelling compared with wt HBc monomer. It is considered that the hydrophobicity of chimeric HBc monomer could be related to the stability of chimeric HBc VLP. The hydrophobicity of HCV core-HBc monomer was determined to decrease significantly compared with wt HBc monomer after inserting HCV core-epitope. The huge change of the hydrophobicity potentially leads to its instability as the hydrophobic interaction between HCV core monomers would be significantly influenced. In contrast, the hydrophobicity of EBNA1-HBc monomer only decreased slightly, and this can explain the similar stability achieved by EBNA1-HBc VLP compared with wt HBc VLP. In addition, molecular dynamic (MD) simulation confirms that HCV core-HBc monomer obtained less stability compared with EBA1-HBc monomer and wt HBc monomer.

Key words: Epstein-Barr virus; Hepatitis C virus; Virus-like particles; Hepatitis B virus core antigen (HBc); Stability; Molecular dynamic (MD) simulation

4.1 Introduction

In **Chapter 3**, two different type of foreign epitopes, Hepatitis C virus core (HCV core) and Epstein-Barr nuclear antigen 1 (EBNA1), were designed and fused into the N-terminus of HBc VLP to form chimeric HCV core-HBc VLP and chimeric EBNA1-HBc VLP. HCV core epitope (aa: 10-53) is composed with 44 amino acids including 18 hydrophilic amino acids and it contains the structure of α-helix, while EBNA1 epitope (aa: 407-417) is composed with 11 amino acid including 3 hydrophilic amino acids and it has no special structure. The expression and purification of two chimeric HBc VLPs were optimized in **Chapter 3**. However, different stability and resistance of the conditions such as pH, buffer composition and temperature were observed during expression, purification and storage of these two chimeric HBc VLPs, especially for HCV core-HBc proteins. HCV core-HBc proteins was found to form irreversible precipitation under some conditions during the optimization of Ammonium Sulphate (AS) and acid precipitation process. It is reported that insertion of different foreign epitopes can have different influence on the stability and assembly of chimeric HBc VLPs by affect the hydrophobic interaction between monomers [1-3].

To further understand the mechanism that cause the change of the stability of chimeric HBc VLPs after insertion of foreign epitopes, computational protein modelling has been developed for the analysis and understand the factors that affect the stability of VLPs. Computational protein modelling is an advanced technique developed to assist the study of the nature of proteins [4]. To effectively applied this technique in the research of the property of chimeric HBc VLPs, the 3D structure of chimeric HBc VLPs

needs to be constructed [5]. Several structure determination methods such as X-ray crystallography [6], cryoEM [7] and NMR [8] have been developed to form the structure files of target proteins. For example, truncated HBc VLP structure was determined using X-ray crystallography to 3.3Å resolution in 1999 [118]. The formed structure files of different proteins can be found in Protein Data Bank (PDB, https://www.rcsb.org/) now. New structure of chimeric VLP proteins either constructed through structure determination methods or by using bioinformatics combined with software such as BIOVIA Discovery Studio [9] and UCSF Chimera [10]. In addition to construct protein structures, Discovery Studio and Chimera can be applied in the study and design of chimeric HBc VLP vaccines. With the software, surface property and bioinformation of the amino acids constituting the VLPs can be achieved. Furthermore, the interaction and stability of VLPs can be analysed using molecular dynamic (MD) simulation technique s [11], and several software such as GROMACS [12], AMBER [13] and NAMD [14] have been employed.

Therefore, in this chapter, two physical stresses, freeze/thaw cycles and temperature, and two chemical stresses, different pH and different concentrations of Sodium Dodecyl Sulphate-Polyacrylamide (SDS), were designed for the stability evaluation of HCV core-HBc VLP and EBNA1-HBc VLP compared with wild type (wt) HBc VLP. Freeze/thaw cycles and temperature were selected to examine the resistance of chimeric HBc VLPs against the change of temperatures. SDS as an ionic detergent containing a long aliphatic chain and a sulphate group was selected to examine the ability of

chimeric HBc VLPs against the electrostatic interaction and hydrophobic interaction. Different pH values were used to examine the pH sensitivity of chimeric HBc VLPs. The wt HBc VLP was used as comparison and evaluate the influence of insertion foreign epitopes on the stability of chimeric HBc VLPs. HPSEC-MALLS was employed to examine the morphology, molecular weight and hydrodynamic radius of chimeric HBc VLPs. In addition, computational protein modelling of the monomers of two chimeric HBc VLPs and wt HBc VLP was performed. The hydrophobicity of monomers of two chimeric HBc VLPs were calculated and compared with the monomer of wt HBc VLP to analyse the influence on the hydrophobic interaction between monomers caused by insertion of foreign epitopes. Finally, to study the impact of insertion of foreign epitopes to HBc sequence on the conformation and the stability of chimeric HBc VLPs, root-mean-square deviation (RMSD) and radius of gyration (Rg) have been applied in the study of the stability of proteins [12, 15]. It is regarded that the smaller the value of RMSD and lower fluctuation level of Rg are the more stable target protein is. This can guide the design of the purification process of chimeric HBc VLPs and save massive experimental time.

4.2. Materials and Methods

4.2.1 Production of chimeric HBc VLP vaccines displaying EBNA1 and HCV core epitope

Chimeric EBNA1-HBc and HCV core-HBc VLPs were expressed and purified with the optimized condition as described in **Chapter 3**.

4.2.2 Characterization of chimeric EBNA1-HBc VLP

Characterization of purified HCV core-HBc and EBNA1-HBc VLP samples were evaluated with, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), High-Performance Size-Exclusion Chromatography (HPSEC) - Multi-Angle Static Laser Light Scattering (MALLS), Intrinsic Fluorescence (IF) and Transmission electron microscopy (TEM).

4.2.2.1 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

12 % SDS-PAGE was prepared for the evaluation of HCV core-HBc and EBNA1-HBc proteins according to the process described in **section 3.2.6.**

4.2.2.2 High-Performance Size-Exclusion Chromatography (HPSEC) - Multi-Angle Static Laser Light Scattering (MALLS)

To further detect the molecular weight of the protein samples, MALLS was applied as it measures the light scattered by a sample into a plurality of angles. In our study, the purity and molecular weight of purified HCV core-HBc and EBNA1-HBc VLP were evaluated by HPSEC-MALLS. HPSEC analysis was performed in Shimadzu UHPLC XR system (Shimadzu, Japan), equipped with TSKgel G4000SWXL column (TOSOH Bioscience, Japan). 50 µl of sample was loaded and eluted at 0.8 ml/min with 50 mM Phosphate buffer (PB) with 100 mM sodium sulphate, pH 7.4. Elution buffer was degassed and filtered by 0.22 µm Millipore membrane (Pall Corporation, USA). The retention time and absorbance at 280 nm were recorded. The HPSEC system was coupled to a Multi-angle laser light scattering (MALLS) Wyatt DAWN® HELEOS II

and Optilab T-rEx (Wyatt Technology, USA) for the detection of the molecular weight. The data were processed with ASTRA software (v. 6.1).

4.2.2.3 Circular Dichroism (CD) Spectroscopy

Circular Dichroism (CD) is an absorption spectroscopy method based on the differential absorption of left and right circularly polarized light and is commonly employed in the evaluation of the secondary structure of protein samples [17]. In our study, difference of secondary structures between HCV core-HBc, EBNA1-HBc VLP and HBc VLP was detected by a Jasco- 810 spectropolarimeter (Jasco, Japan), using a quartz cuvette with 0.1 cm pathlength. All the protein samples were prepared at the concentration of 0.4 mg/ml in 20 mM PB (pH 7.4). The baseline of buffer was subtracted from the experimental spectra for corrections. The reported CD spectra are the average of three scans.

4.2.2.4 Intrinsic Fluorescence (IF)

Intrinsic fluorescence is a type of electromagnetic spectroscopy using ultraviolet light (UV light) to excite the electrons in molecules of proteins [18]. IF spectroscopy can be applied to evaluate the conformational state of protein samples leading to the tryptophan fluorescence shifts [19]. HCV core-HBc, EBNA1-HBc and HBc VLPs were analysed using Hitachi F-4500 fluorescence spectrofluorometer (Hitachi, Japan) using a quartz cell of 1.0 cm path length. The emission spectra were excited at 280 nm with a slit width of 5.0 nm and recorded between 300 and 400 nm. The measurement was obtained at a protein concentration of 0.2 mg/ml in PBS buffer (pH 7.4) at 25 °C.

4.2.2.5 Transmission electron microscopy (TEM).

TEM of EBNA1-HBc and HCV core-HBc was taken as described in **section 3.2.7**.

4.2.3 Stability evaluation of chimeric HBc VLPs

The stability of chimeric EBNA1-HBc and HCV core-HBc VLP was examined and compared with wt HBc VLP under different stresses including temperatures, freeze/thaw cycles, sodium dodecyl sulphate (SDS) and pH. The molecular weight and hydrodynamic radius (Rh) of dialyzed samples were examined using HPSEC-MALLS analysis. Chimeric HBc VLP samples after treating with different stresses were centrifuged at 10,000 rpm for 30 min and the concentration of the supernatant of each sample was examined using Bradford assay [21]. The concentration of dialyzed samples was adjusted to 1 mg/ml using 20 mM PB, pH 7.4 before the stability evaluation under different stresses. The wt HBc VLP at 1mg/ml was used as reference. For temperature assay, chimeric HBc VLP samples were placed in the water bath for 30 min at the designed temperature (25 ºC, 50 ºC, 70 ºC, 80 ºC and 90 ºC). After treatment, samples were stored at 4 °C and analysis was conducted within 4 h. For freeze/thaw cycle assay, chimeric HBc VLP samples were stored in -20 °C overnight and thaw at 25 °C in a water bath for 4 different cycles. For SDS resistance assay, chimeric HBc VLP samples were incubated for 30 min in SDS-Tris-HCl buffer, pH 7.4 at the given concentrations (w/v) including 0.05 %, 0.06 %, 0.07 % and 0.08 % before analysis. For pH stability assay, chimeric HBc VLP samples were incubated with different buffers at designed pH values including pH 11.0, pH 9.0, pH7.5, pH 5.0 and pH 2.0 for 4 h before the analysis. The pH of the buffers was measured at an error

within 0.2.

4.2.4 Computational simulation of chimeric HBc VLPs

To understand and investigate the possible causes for the differences of chimeric HBc VLPs after insertion of foreign epitopes, atomic structure of HBc (PDB ID: 3J2V) was employed. EBNA1 epitope (HPVGEADYFEY) form EBNA1 protein (PDB ID: 5WMF) and HCV core epitope (KTKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGP RLGVRATRKTS) from HCV core protein (PDB ID: 1NLB) were added to the N-terminus of HBc to form the atomic structure of chimeric EBNA1-HBc and HCV core-HBc using BIOVIA Discovery Studio. Due to the limitation of the calculation power of the computer, bioinformation of EBNA1-HBc monomer, HCV core-HBc monomer and HBc monomer were investigated. Hydrophobicity of whole monomer and 5-residue running average hydrophobicity representing the hydrophobicity of an area was calculated with the data from BIOVIA Discovery Studio. MD simulations were performed using GROMACS 2018.5 using GROMOS96 43a1 force field. SPC/E water model in a cubic box with a distance of 1.0 nm was applied for simulation and system was neutralized with Na and Cl ions. Energy minimization was performed using the steepest descent algorithm with a maximum force constraint of 1000.0 kJ/mol/nm. Particle mesh Ewald (PME) method and Linear Constraint Solver (LINCS) algorithm was employed to calculate and constrain bonds. Temperature equilibration was performed at 300 K for 100 ps with the V-rescale method. Then, the pressure was equilibrated with the pressure coupling of Parrinello–Rahman for 100 ps. Finally, MD simulations were run for 3ns with 2-fs time steps. The trajectories of the MD

simulations were analysed for root-mean-square deviation (RMSD) and the RMSD data was visualized with Origin 9.

4.3 Results and discussion

4.3.1 Conformation characterization of chimeric HBc VLPs after purification

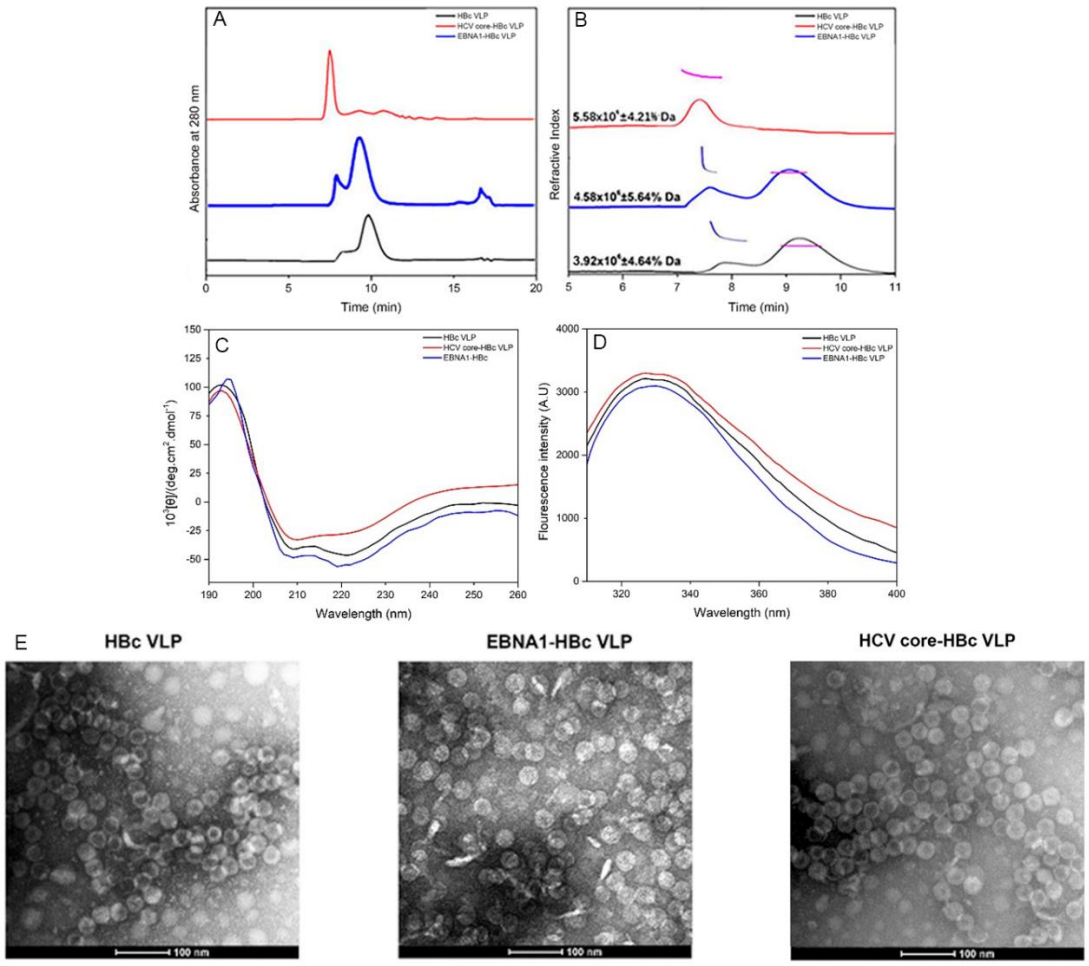

Figure 4.1 HPSEC (A) -MALLS (B) of the purified EBNA1-HBc and HCV core-HBc on TSK G4000 SWXL column. (pink line: molecular weight) Characterization of chimeric HBc VLP including CD spectra(C), IF spectra (D) and TEM analyses (E).

Reports have indicated that the insertion of foreign epitopes to HBc VLP proteins can potentially decrease the stability of VLP structure or inhibit the assembly of HBc into VLP structure due to the nature of foreign epitopes [22-24]. To further evaluate whether

the developed purification process is suitable to produce chimeric HBc VLPs in correctly assembled VLP structure, the purified EBNA1-HBc VLP and HCV core-HBc VLP were characterized using HPSEC, CD, IF and TEM, the conformation of VLPs was compared with HBc VLP.

As is shown in **Figure 4.1A** and **B,** purified chimeric EBNA1-HBc VLP showed similar morphology compared with HBc VLP. HPSEC-MALLS is often used for the separation and analysis of protein sample according to their molecular weights [25, 26]. The molecular weight of chimeric EBNA1-HBc VLP was around 4.58×10^6 Da, which is close to the theoretical molecular weight of 3.97×10^6 Da (T=3) and 5.29×10^6 Da (T=4). The detected molecular weight of expressed HCV core-HBc VLP was around 5.58×10^6 Da which is within the theoretical molecular weight for HCV core-HBc VLP (4.67×10^6 Da (T=3) or 6.23×10^6 Da (T=4)). This suggested that chimeric HBc VLPs were expressed in a mixture of T=3 and T=4 particles. Small amount of aggregation (around 15 %) of both chimeric EBNA1-HBc VLP and HBc VLP were observed in HPSEC-MALLS results. However, in the HPSEC result, only one fraction peak was detected. This phenomenon could be explained by limitation of TSKgel G4000SWXL column which cannot separate between the aggregation peak and the VLP peak of HCV core-HBc. The small amount of aggregation of chimeric HBc VLP could result from the aggregation behaviour from HBc VLP due to the solution environment such pH and the buffering agent [27]. For example, Schumacher et.al. (2018) claimed that self-aggregation of HBc VLP was formed over time in Tris-buffer [1].

The secondary structures of the HBc-VLPs, chimeric EBNA1-HBc VLP and HCV

core-HBc VLP were examined by (CD) spectroscopy analysis. As is shown in **Figure 4.1C**, in comparison to HBc VLP, the peak at 222 nm which represented the α-helix structure of chimeric EBNA1-HBc VLP was negligible impacted after insertion of EBNA1 epitope. The increase of the ellipticity value at 222nm of chimeric EBNA1-HBc VLP suggests a structurally increase in α-helix content after the insertion of foreign EBNA1 epitope to HBc VLP. On the other hand, HCV core-HBc VLP had a large decrease of ellipticity value at peak of 222 nm in CD measurement, corresponding to the change of α-helix structure. This suggests that the α-helix structure on HCV core epitope could influence the formation of α-helix in chimeric HCV core-HBc, leading to the reduce of the ratio of α-helix. In the fluorescent measurement of EBNA1-HBc VLP, HCV core-HBc VLP and HBc VLP shown in **Figure 4.1D**, same fluorescence maximum emission wavelengths were observed. This suggests that there is no significant impact of the EBNA1 epitope and HCV core epitope on the tertiary structure of VLPs. The integrity of chimeric EBNA1-HBc VLP and HCV core-HBc VLP were characterized by TEM. TEM image in **Figure 4.1E** demonstrates that the produced EBNA1-HBc and HCV core-HBc proteins obtained the same VLP structure as HBc VLP.

4.3.2 Stability evaluation of chimeric HBc VLPs

Stable VLP structure of chimeric VLP-based vaccine is essential to ensure its immunogenicity *in vivo*. The stability of expressed chimeric EBNA1-HBc VLP and HCV core-HBc VLP was evaluated under different stresses including temperature, freeze/thaw storage cycles, SDS and pH values, and wt HBc VLP was examined in the

same conditions as reference. In the evaluation, HCV core-HBc VLP was found to be sensitive to the stresses and formed irreversible precipitation. **Table 4.1** illustrates the concentrations of supernatants of HCV core-HBc protein samples after being treated with different stresses. HCV core-HBc VLP was found to be sensitive to pH value and SDS. All HCV core-HBc VLP formed irreversible precipitation with 0.05 % or higher concentration of SDS, while it was only stable at around pH 7.5. When treating with physical stresses, HCV core-HBc showed good stability for freeze/thaw cycles assay as it remained concentration of 0.87 mg/ml, and 0.56 mg/ml HCV core-HBc VLP proteins left when testing with temperature of 50 ºC. However, the stability of HCV core-HBc VLP is much lower than EBN1-HBc and wt HBc VLP, in general.

Table 4.1 Concentrations of supernatant of chimeric HBc VLP samples after being treated with different stresses

Stress	**Conditions**	**$C_{HCV\ core\text{-}HBc}$ (mg/ml)**	**$C_{EBNA1\text{-}HBc}$ (mg/ml)**	**$C_{wt\ HBc}$ (mg/ml)**
Temperature	25 ºC	1	1	1
	50 ºC	0.56	0.84	0.91
	70 ºC	0	0.86	0.87
	80 ºC	0	0.53	0.62
	90 ºC	0	0	0
Freeze/thaw cycles	0	1	1	1
	1	0.96	1	1
	2	0.91	0.96	0.97
	3	0.92	0.97	0.97
	4	0.87	0.85	0.96
SDS	0.00 %	1	1	1
	0.05 %	0	0.41	0.74

	0.06 %	0	0	0.42
	0.07 %	0	0	0
	0.08 %	0	0	0
pH value	11	0	0.55	0.31
	9	0.36	0.84	0.91
	7.5	1	1	1
	5	0	0	0.74
	2	0	0.35	0.21

Since HCV core-HBc showed poor stability and formed irreversible precipitation in most cases. HPSEC-MALLS results for detection of morphology, molecular weight and Rh of EBNA1-HBc wt and wt HBc VLP samples were presented. As shown in **Figure 4.2**, chimeric EBNA1-HBc VLP was not able to maintain the VLP structure when it was heated at 80 °C or higher temperatures. Compared with HBc VLP, chimeric EBNA1-HBc VLP showed a similar thermal stability. Also, the molecular weight and Rh measurement of EBNA1-HBc VLP under different temperatures were similar to wt HBc VLP. In addition, when temperature reached to 80 °C, the VLP and aggregation peaks in HPSEC of both EBNA1-HBc VLP and wt HBc VLP were disappear while the protein concentration, molecular weight and Rh can all be detected with large error bar. It suggests that EBNA1-HBc VLP and wt HBc VLP can form large soluble aggregations and may be blocked at the entry of HPLC column.

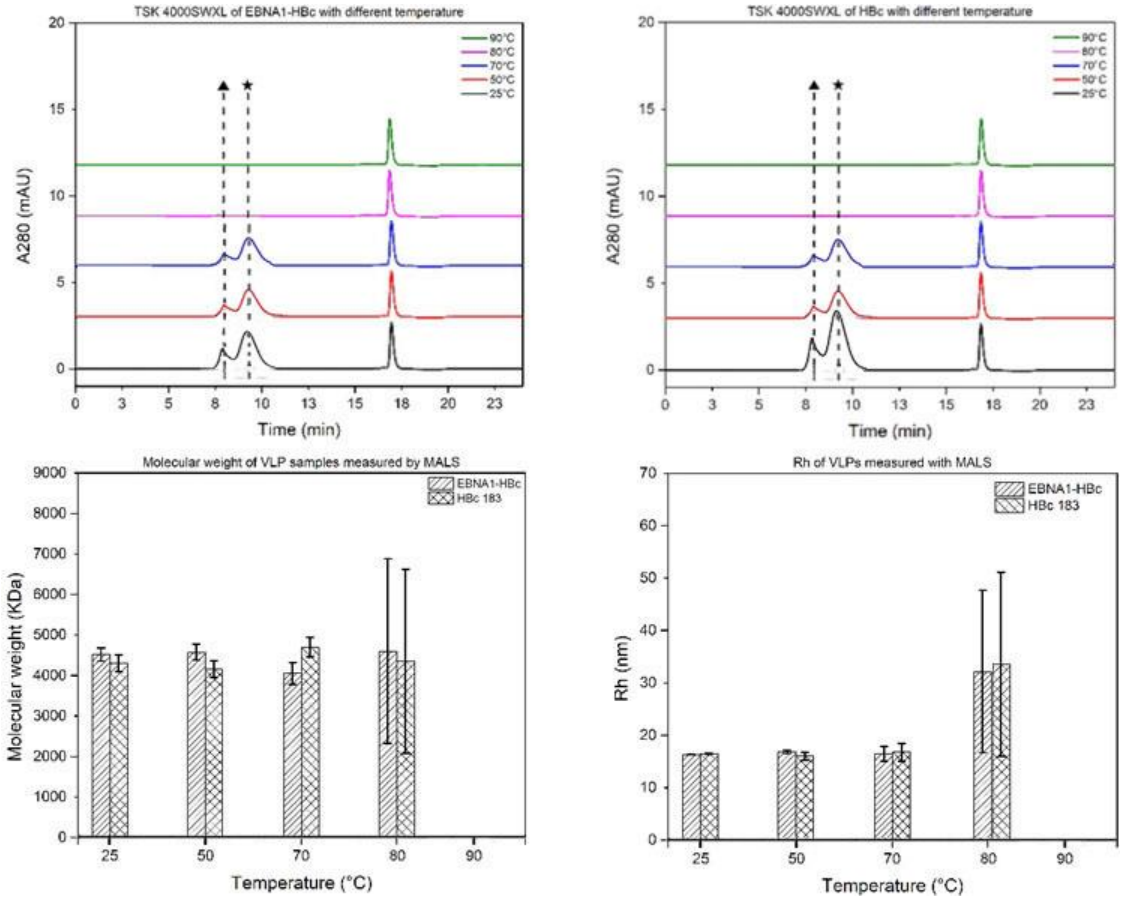

Figure 4.2 Effects of temperature on VLPs stability. HPLC of EBNA1-HBc VLPs (A) and HBc 183 (B). Molecular weight (C) and Rh (D) of EBNA1-HBc VLPs and HBc 183 (★).

To test the stability of chimeric EBNA1-HBc VLP in the freezing storage condition, various numbers of repeated freeze/thaw cycles on EBNA1-HBc VLP were conducted. As shown in **Figure 4.3**, EBNA1-HBc VLP showed similar morphology, molecular weight and Rh in freeze/thaw cycles assay compared with wt HBc VLP. However, in the fourth cycle, an increased particle size of EBNA1-HBc VLP (Rh of 29.5 nm) was detected and a slightly lower protein concentration was detected for 0.85 mg/ml (shown in **Table 4.1**) compared with HBc VLP. The achieved results indicate that the insertion of EBNA1-HBc VLP showed minor impact on the structure of chimeric EBNA1-HBc VLP under the physical stress.

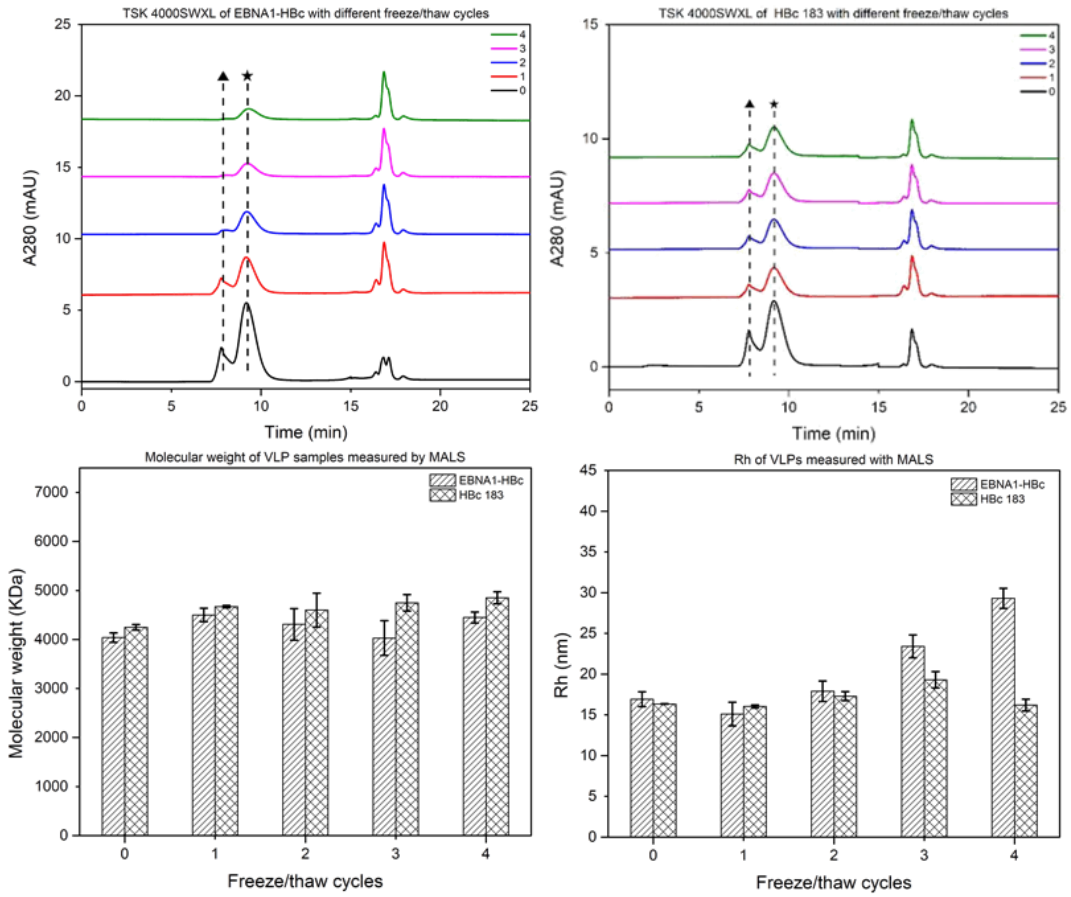

Figure 4.3 Effects of freeze/thaw cycles on VLPs stability. HPLC of EBNA1-HBc VLPs (A) and HBc 183 (B). Molecular weight (C) and Rh (D) of EBNA1-HBc VLPs and HBc 183 (★).

For further evaluation of the stability of EBNA1-HBc VLP, two chemical stresses including treating with different concentration of SDS and pH values were examined. As shown in **Figure 4.4**, the influence of an ionic surfactant agent (SDS) at concentrations between 0.01 and 0.08 % on the stability of chimeric EBNA1-HBc VLP and wt HBc VLP was analysed. Molecular weight and Rh results indicate that EBNA1-HBc VLP was able to maintain the VLP structure with lower than 0.05 % of SDS while HBc VLP can still keep VLP structure at 0.06 % of SDS. This matches with the results measured with HPSEC-MALLS that all peaks disappeared in chimeric EBNA1-HBc VLP when concentration of SDS was higher than 0.06 %. In addition, a broad pH range was applied in the testing of the stability of chimeric EBNA1-HBc VLP and wt HBc

VLP. As shown in **Figure 4.5**, HBc VLP showed a better resistance at lower pH value (pH=5) than EBNA1-HBc VLP and EBNA1-HBc VLP was stable between pH 7.5 to pH 11. In addition, the protein concentration of wt HBc VLP at pH 11 (0.31 mg/ml) was slightly lower than that of EBNA1-HBc VLP (0.5 mg/ml). After insertion of EBNA1 epitope, stronger influence on the stability of chimeric VLP under chemical stress than that on physical stress was detected in the evaluation.

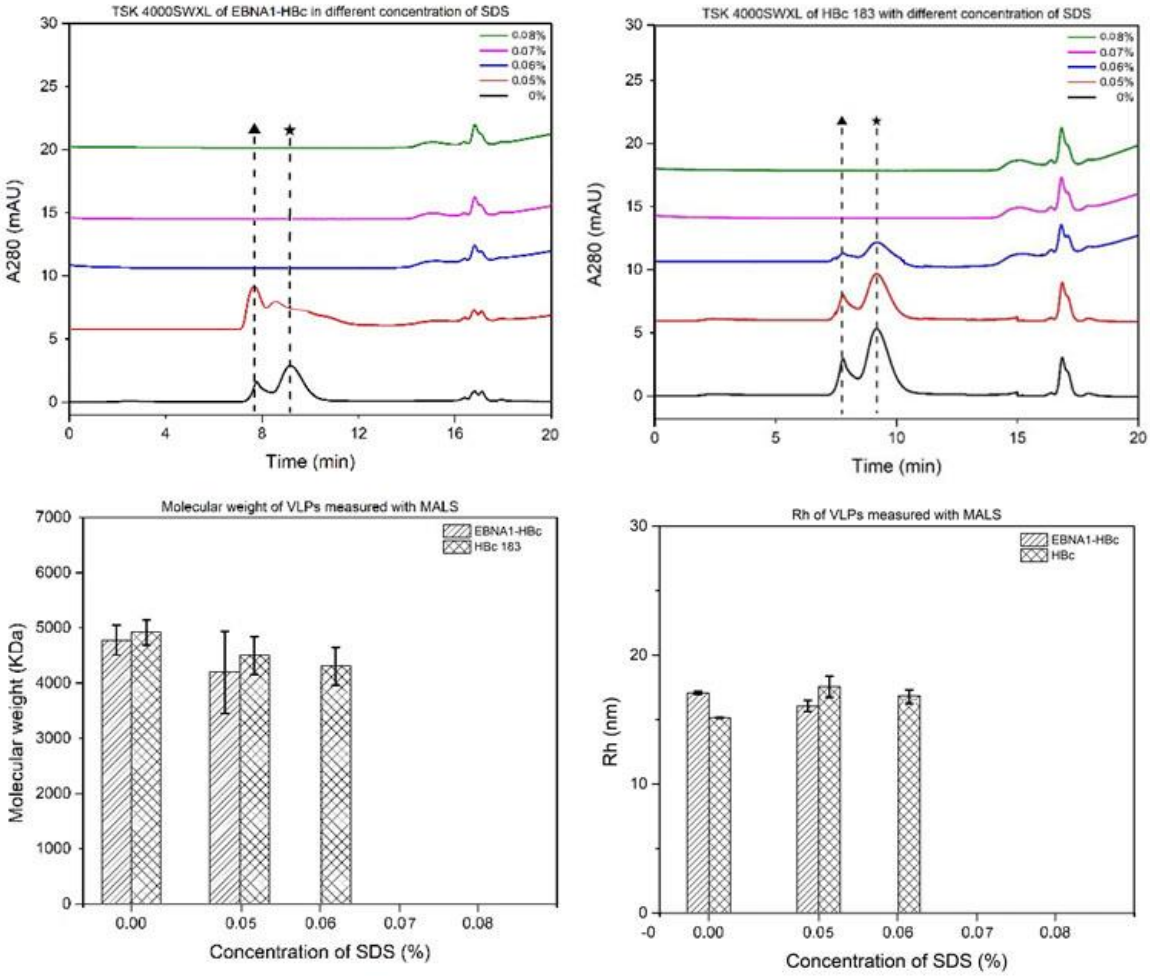

Figure 4.4 Effects of SDS on VLPs stability. HPLC of EBNA1-HBc VLPs (A) and HBc 183 (B). Molecular weight (C) and Rh (D) of EBNA1-HBc VLPs and HBc 183 (★).

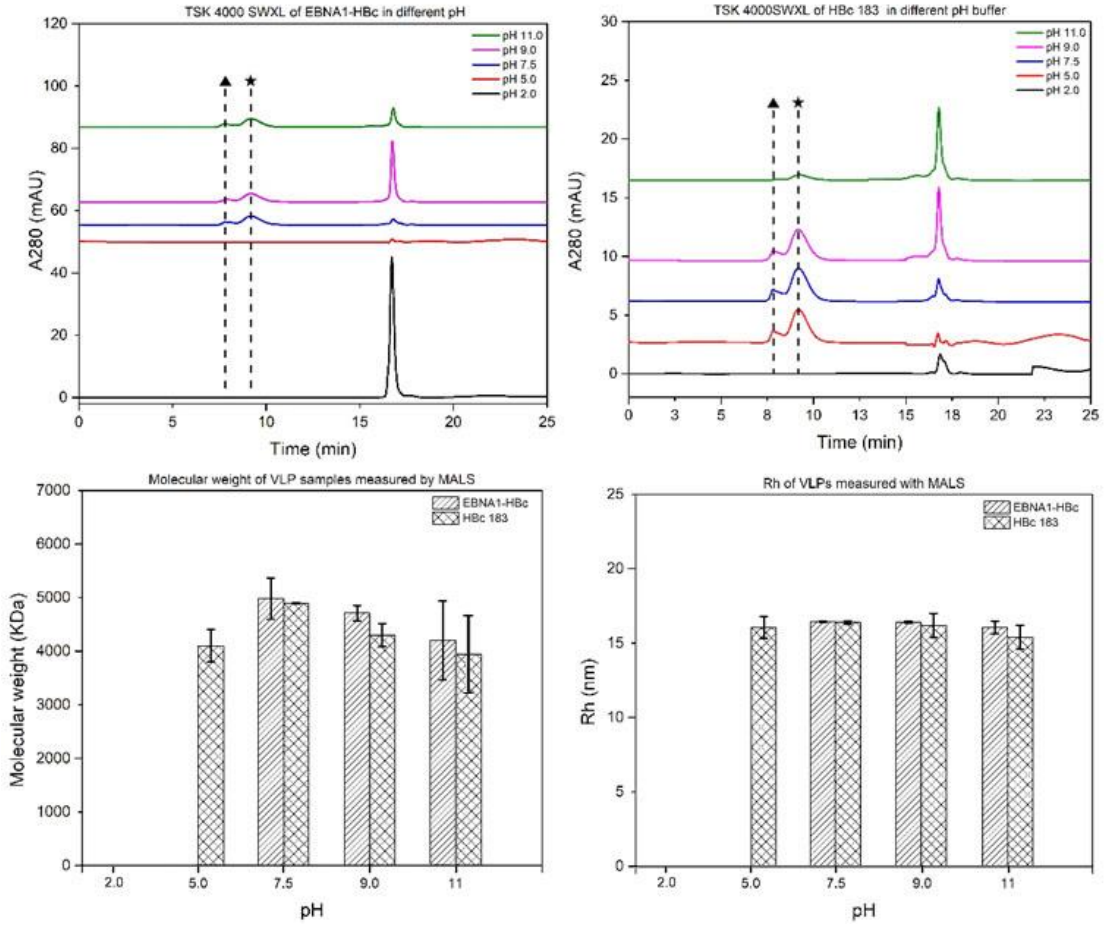

Figure 4.5 Effects of pH on VLPs stability. HPLC of EBNA1-HBc VLPs (A) and HBc 183 (B). Molecular weight (C) and Rh (D) of EBNA1-HBc VLPs and HBc 183 (★).

4.3.3 Molecular Dynamic (MD) simulation to predict protein stability after insertion of different epitopes

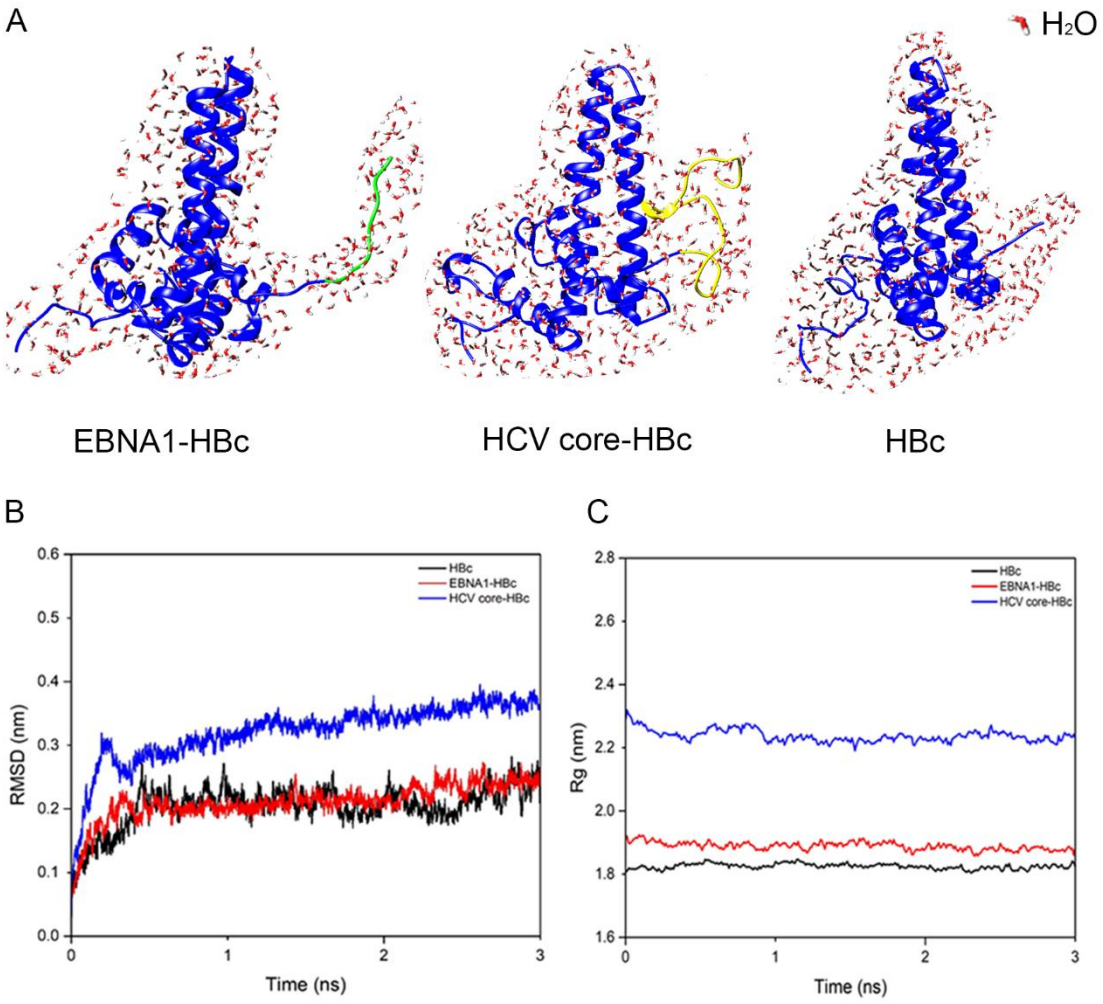

Figure 4.6 3D image of chimeric HBc VLP monomers with water shell after MD simulation (A). Plot of RMSD (B) and radius of gyration (C) of HBc, EBNA1-HBc and HCV core-HBc monomer running with GROMACS in CHARMM force field in water environment. HBc in blue, EBNA1 in green and HCV core in yellow.

Insertion of foreign antigens to HBc VLP could have impacts on their stability which would then influence the purification process. To predict the stability of chimeric HBc VLP after insertion of foreign epitopes and assist the design for purification process for removal of HCPs, Molecular Dynamic simulation (MD simulation) was applied to study the interaction between chimeric HBc VLP monomers and water molecule

(**Figure 4.6A**). Data of RMSD and Rg of EBNA1-HBc, HCV core-HBc and HBc was processed and plot into graphics. As is shown in **Figure 4.6A**, EBNA1-HBc showed similar backbone RMSD compared with HBc while HCV core-HBc had a higher RMSD, it indicates that HCV core-HBc had a lower stability in water environment compared with EBNA1-HBc and HBc. In **Figure 4.6B**, the radius of gyration of HCV core-HBc fluctuates about 0.2 nm higher than the fluctuation level of EBNA1-HBc and HBc (less than 0.1 nm). The RMSD and Rg results indicated that HCV core-HBc is less stable than other two samples in the water environment, which indicating that HCV core-HBc VLP potentially had weaker interaction with water molecules in the environment. This finding potentially suggested that HCV core-HBc was more hydrophobic, comparing with EBNA1-HBc in theory.

The value of hydrophobicity (**Figure 4.7**) and 5-residue running average hydrophobicity (**Figure 4.8**) of the monomers of HCV core-HBc, EBNA1-HBc and HBc were calculated using Discovery Studio. As shown in **Figure 4.9**, the total value of hydrophobicity of monomer of HCV core-HBc turned to negative (-7.3) which indicates that after insertion HCV core epitope, its surface turns to a hydrophilic potential, while the hydrophobicity of EBNA1-HBc monomer (5.6) decreased but remained positive compared with wt HBc monomer (14.3).

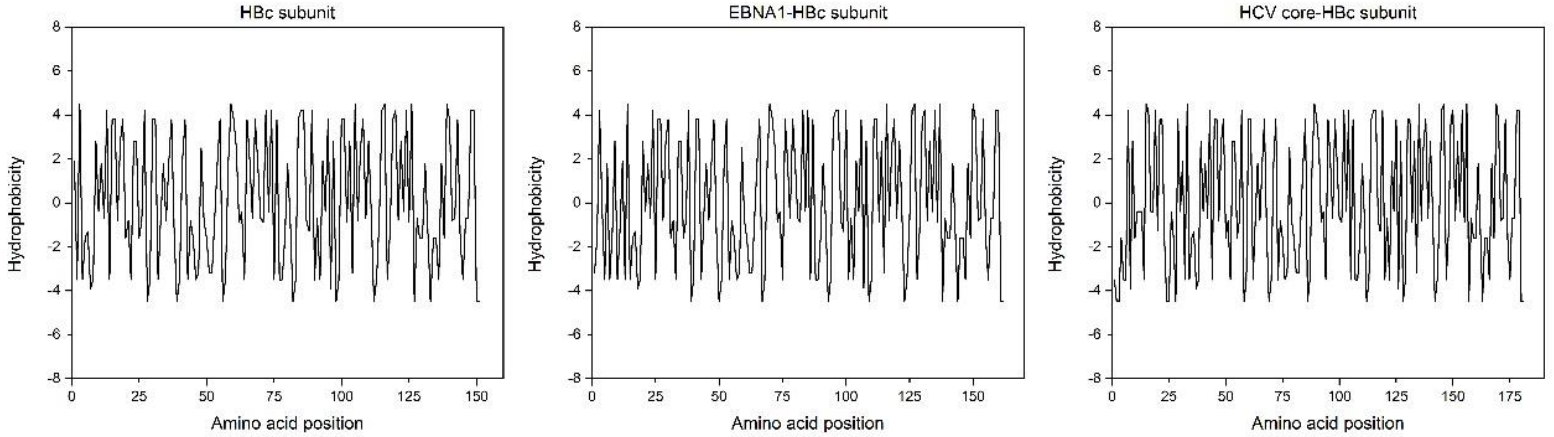

Figure 4.7 hydrophobicity of amino acids in HBc, EBNA1-HBc and HCV core-HBc monomer.

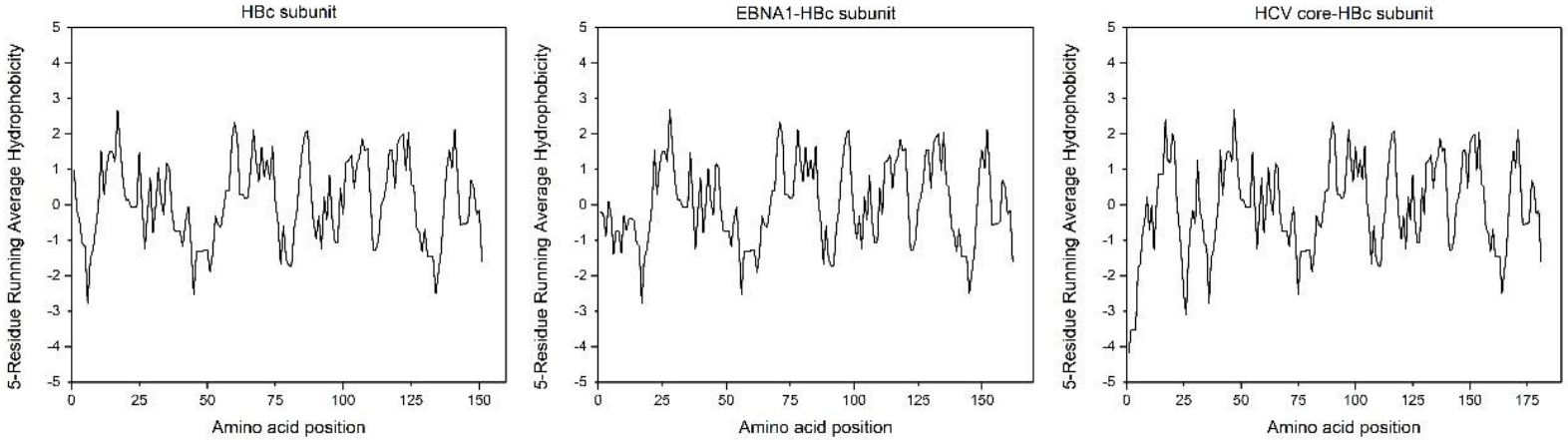

Figure 4.8 Plot of 5-Residue Running Average Hydrophobicity of amino acids in HBc, EBNA1-HBc and HCV core-HBc monomer.

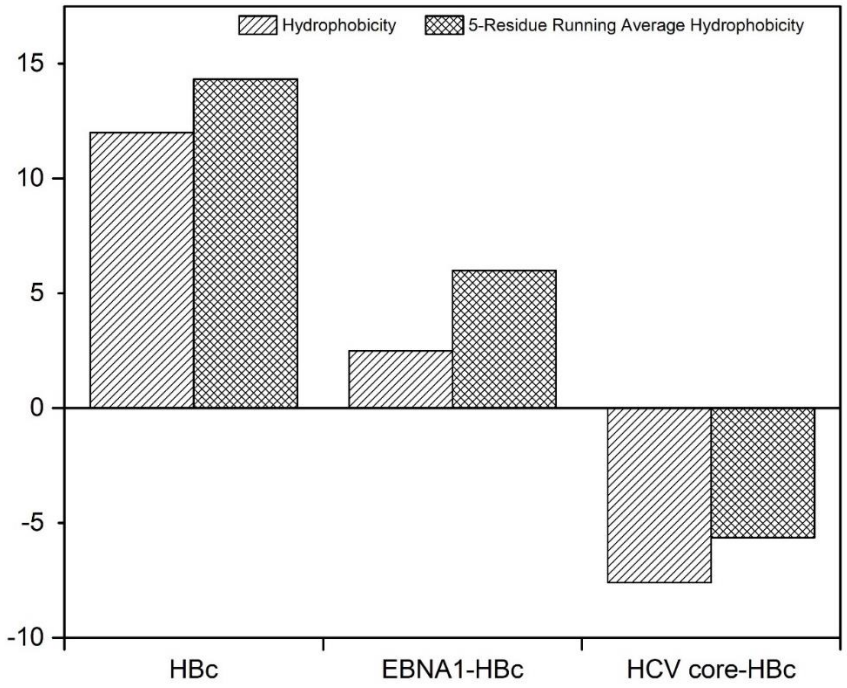

Figure 4.9 Hydrophobicity and 5-Residue Running Average Hydrophobicity of HBc, EBNA1-HBc and HCV core-HBc monomer.

On the other hand, the value of hydrophobicity and 5-residue running average hydrophobicity of EBNA1-HBc reduced compared with HBc but EBNA1-HBc monomer remained hydrophobic. This could be the reason why it has a minor influence on the stability of chimeric EBNA1-HBc under the physical stress, but when treating with SDS, whose mechanism is to disturb the hydrophobic bond in the proteins, reduced resistance of EBNA1-HBc VLP was detected compared with wt HBc VLP.

4.4 Discussion

In our project, two different types of epitopes, short and non-structural EBNA1 epitope and long and structural HCV core epitope were fused with HBc VLP to form recombinant chimeric HBc VLPs. HCV core and EBNA epitopes were designed to fused to the N-terminal of HBc VLP to be exposed on the surface of HBc VLP to induce

strong immune response. The soluble expression yields in *E. coli* expression system of these two chimeric HBc VLPs were optimized, and the purification process was also optimized. However, during the optimization process, irreversible aggregation and precipitation of HCV core-HBc VLP proteins was detected. In addition, these precipitation and aggregation could be related to several conditions such as pH value, buffer composition and temperature. Since stable VLP structure of chimeric HBc VLP is essential to ensure its *in vivo* immunogenicity, stabilities of produced HCV core-HBc VLP and EBNA1-HBc VLP were evaluated in this project.

One apparent influence caused by the insertion of foreign epitope reported by Karpenko et al., (2000) is that it can affect the appropriate conformation of chimeric HBc VLP and prevent the formation of correctly assembled VLP structure [22]. The characterization results indicate that the insertion of long and structural foreign epitopes, HCV core, has a major impact on the secondary structure, but a minor impact on the tertiary structure and conformation. By contrast, EBNA1 made negligible impact on all levels of HBc VLP structure. The change on the secondary structure of HCV core-HBc VLP, compared with EBNA1-HBc VLP and HBc VLP, could potentially cause the difference in the stability of EBNA1-HBc VLP and HCV core-HBc VLP. Aside this, the tertiary structure and TEM image results of HCV core-HBc and EBNA1-HBc proves that the developed downstream process has negligible impact on the assembly structure of both chimeric HBC VLPs which could keep the antigenicity of both chimeric HBc VLPs.

Then different physical and chemical stresses were applied to examine the ability of chimeric HBc VLP to maintain the assembly of VLP structure. In the stability evaluation, HCV core-HBc VLP shows lower stability to resist the stresses compared with EBNA1-HBc VLP and wt HBc VLP. This could come from the nature and property of HCV core epitope as it is a long and structural epitope. In addition, for EBNA1-HBc, it obtains similar stability under physical stress compared with wt HBc VLP, while when applying chemical stresses, it shows lower stability compared with wt HBc VLP. Its resistance limit for SDS is 0.05 % while the resistance limit of wt HBc VLP for SDS is 0.06. SDS is an ionic detergent containing a long aliphatic chain and a sulphate group. Its hydrophobic tail can interact with hydrophobic groups in the proteins, which can influence the assembly of chimeric HBc VLPs, as the formation of VLP structure is an entropy-driven process relying on hydrophobic interaction [28]. The decreased tolerance of EBNA1-HBc VLP to SDS suggests that the insertion of EBNA1 epitope to HBc VLP affect the hydrophobic conformation and makes it easier to be interacted by SDS. In addition, the stability of EBNA1-HBc VLP in low pH environment (pH 5) was also lower than wt HBc VLP.

To understand the mechanism behind the decreased stability of chimeric HBc VLPs compared with wt HBc VLP, computational protein modelling was applied. To perform the protein modelling, several stages are included such as 1) construction of protein structures, 2) analysis of the properties of constructed protein molecules including charge, hydrophobicity and chemical bonds, and 3) stability and interaction evaluation

using Molecular Dynamic (MD) simulation [5]. In our project, the protein structures of HCV core-HBc monomer, EBNA1-HBc monomer and wt HBc monomer were generated from the protein structures from PDB. The constructed chimeric HBc monomers were evaluated using BIOVIA Discovery Studio. Results indicate that after the insertion of EBNA1 and HCV core, the hydrophobicity of chimeric HBc monomers decreased. Since hydrophobic interaction is vital for the assembly of HBc VLP structure, the decreased hydrophobicity of the monomers can largely influence the hydrophobic interaction between monomers and further impact on the stability and assembly of chimeric HBc VLPs. For HCV core-HBc VLP, the calculated hydrophobicity of HCV core-HBc monomer turns to be negative which can explain its sensitivity to the minor changes of the environment as it can be in a fragile condition. The different hydrophobicity of HCV core-HBc and EBNA1-HBc monomers confirms that the impact of the insertion of foreign antigens comes from the nature of antigens, and the hydrophobicity of chimeric HBc monomer can be used to reflect the stability of chimeric HBc VLP. In addition, RMSD and Rg results confirms our findings that HCV core-HBc was less stable in the water environment compared with EBNA1-HBc and HBc. These findings in computational protein modelling can be used to guide the design of chimeric HBc VLP and optimize the purification and storage conditions to maintain its assembled VLP structure.

4.5 Conclusion

To understand and investigate the influence of the insertion of different foreign epitopes

to HBc VLP on the stability, short and non-structured EBNA1 epitope and long and structured HCV core epitopes were employed in our study and two chimeric EBN1-HBc VLP and HCV core-HBc VLP were expressed and purified for the evaluation. It is found that insertion of HCV core epitope had impact on the secondary structure of HCV core-HBc which potentially is the reason for the less stability of HCV core-HBc, compared with EBNA1-HBc and HBc. Aside this, the tertiary structure and TEM image results confirm that the produced chimeric HBc VLPs are in correctly assembled VLP structure. In the stability evaluation, HCV core-HBc VLP is found to be sensitive and less stable than EBNA1-HBc VLP as minor changes such as temperature and buffer condition can lead to irreversible precipitation. EBNA1-HBc VLP shows good stability under physical stresses while the stability dropped when applying chemical stresses like low pH and high concentration of SDS. According to the protein modelling results, a possible explanation for this phenomenon is that after the insertion of HCV core epitope to HBc VLP, the hydrophobicity of HCV core-HBc monomer significantly drops as well as the hydrophobic interaction between monomers, leading to a relatively fragile HCV core-HBc VLP assembly. In contrast, after insertion of EBNA1 epitope, only a minor change of hydrophobicity of EBNA1-HBc monomer was found compared with wt HBc monomer. The slightly changed surface property can explain the minor influence on the morphology of chimeric EBNA1-HBc VLP compared with HBc VLP. However, the reduced hydrophobicity could cause the lower resistance against protein surfactant reagent due to the reduced hydrophobic interaction between monomers. In

addition, RMSD and Rg results indicate that after insertion of long and structural HCV core epitope, HCV core-HBc was less stable in the water environment compared with EBNA1-HBc and HBc without insertion of foreign epitopes. These findings could be beneficial to the design and production of chimeric HBc VLP-based vaccines. However, further computational protein simulation evaluations such as the binding energy and interaction between the monomers and VLP structures of chimeric HBc VLP-based vaccines are needed for the design and development of HBc VLP vaccine platform.

References

[1] J. Schumacher, T. Bacic, R. Staritzbichler, M. Daneschdar, T. Klamp, P. Arnold, S. Jagle, O. Tureci, J. Markl, U. Sahin, Enhanced stability of a chimeric hepatitis B core antigen virus-like-particle (HBcAg-VLP) by a C-terminal linker-hexahistidine-peptide, J Nanobiotechnology 16(1) (2018) 39.

[2] C.G. Alexander, M.C. Jürgens, D.A. Shepherd, S.M. Freund, A.E. Ashcroft, N. Ferguson, Thermodynamic origins of protein folding, allostery, and capsid formation in the human hepatitis B virus core protein, Proceedings of the National Academy of Sciences 110(30) (2013) E2782-E2791.

[3] Y. Lu, W. Chan, B.Y. Ko, C.C. VanLang, J.R. Swartz, Assessing sequence plasticity of a virus-like nanoparticle by evolution toward a versatile scaffold for vaccines and drug delivery, Proceedings of the National Academy of Sciences of the United States of America 112(40) (2015) 12360-12365.

[4] L. He, J. Zhu, Computational tools for epitope vaccine design and evaluation, Curr. Opin. Virol. 11 (2015) 103-112.

[5] L. Zhang, L.H.L. Lua, A.P.J. Middelberg, Y. Sun, N.K. Connors, Biomolecular engineering of virus-like particles aided by computational chemistry methods, Chem. Soc. Rev. 44(23) (2015) 8608-8618.

[6] M.S. Smyth, J.H. Martin, x ray crystallography, Molecular pathology : MP 53(1) (2000) 8-14.

[7] K. Murata, M. Wolf, Cryo-electron microscopy for structural analysis of dynamic biological macromolecules, Biochimica et Biophysica Acta (BBA) - General Subjects 1862(2) (2018) 324-334.

[8] A. Cavalli, X. Salvatella, C.M. Dobson, M. Vendruscolo, Protein structure determination from NMR chemical shifts, Proceedings of the National Academy of Sciences 104(23) (2007) 9615-9620.

[9] M.A. Khan, M.U. Hossain, S.M. Rakib-Uz-Zaman, M.N. Morshed, Epitope-based peptide vaccine design and target site depiction against Ebola viruses: an immunoinformatics study, Scand J Immunol 82(1) (2015) 25-34.

[10] M.M. Pourseif, M. Yousefpour, M. Aminianfar, G. Moghaddam, A. Nematollahi, A multi-method and structure-based in silico vaccine designing against Echinococcus granulosus through investigating enolase protein, BioImpacts : BI 9(3) (2019) 131-144.

[11] J. Zheng, L. Zhang, R. Tang, S. Bai, N.K. Connors, L.H.L. Lua, Y.P. Chuan, A.P.J. Middelberg, Y. Sun, Energetic Changes Caused by Antigenic Module Insertion in a Virus-Like Particle Revealed by Experiment and Molecular Dynamics Simulations, PLoS One 9(9) (2014).

[12] S. Mobini, M. Chizari, L. Mafakher, E. Rismani, E. Rismani, Computational Design of a Novel VLP-based Vaccine for Hepatitis B Virus, Front. Immunol. 11(2074) (2020).

[13] I. Cielens, L. Jackevica, A. Strods, A. Kazaks, V. Ose, J. Bogans, P. Pumpens, R. Renhofa, Mosaic RNA phage VLPs carrying domain III of the West Nile virus E protein, Mol. Biotechnol. 56(5) (2014) 459-69.

[14] J.R. Perilla, K. Schulten, Physical properties of the HIV-1 capsid from all-atom molecular dynamics simulations, Nature Communications 8(1) (2017) 15959.
[15] L. Zhang, R. Tang, S. Bai, N.K. Connors, L.H. Lua, Y.P. Chuan, A.P. Middelberg, Y. Sun, Energetic changes caused by antigenic module insertion in a virus-like particle revealed by experiment and molecular dynamics simulations, PLoS One 9(9) (2014) 107313.
[16] R.B. Gupta, K. Shepherd, Two-step one-dimensional SDS-PAGE analysis of LMW subunits of glutelin, Theoretical and Applied Genetics 80(1) (1990) 65-74.
[17] N.J. Greenfield, Using circular dichroism spectra to estimate protein secondary structure, Nat. Protoc. 1(6) (2006) 2876-2890.
[18] S. Gorinstein, I. Goshev, S. Moncheva, M. Zemser, M. Weisz, A. Caspi, I. Libman, H.T. Lerner, S. Trakhtenberg, O. Martín-Belloso, Intrinsic tryptophan fluorescence of human serum proteins and related conformational changes, Journal of protein chemistry 19(8) (2000) 637-642.
[19] J.T. Vivian, P.R. Callis, Mechanisms of Tryptophan Fluorescence Shifts in Proteins, Biophysical Journal 80(5) (2001) 2093-2109.
[20] H. Arakawa, K. Umemura, A. Ikai, Protein images obtained by STM, AFM and TEM, Nature 358(6382) (1992) 171-173.
[21] Y. Zhang, Y. Liu, B. Zhang, S. Yin, X. Li, D. Zhao, W. Wang, J. Bi, Z. Su, In vitro preparation of uniform and nucleic acid free hepatitis B core particles through an optimized disassembly-purification-reassembly process, Protein Expression and Purification (2020) 105747.
[22] L. Karpenko, V. Ivanisenko, I. Pika, N. Chikaev, A. Eroshkin, T. Veremeiko, A. Ilyichev, Insertion of foreign epitopes in HBcAg: how to make the chimeric particle assemble, Amino Acids 18(4) (2000) 329-337.
[23] M. Vogel, J. Vorreiter, M. Nassal, Quaternary structure is critical for protein display on capsid-like particles (CLPs): Efficient generation of hepatitis B virus CLPs presenting monomeric but not dimeric and tetrameric fluorescent proteins, Proteins: Structure, Function, and Bioinformatics 58(2) (2005) 478-488.
[24] E.A. Blokhina, V.V. Kuprianov, L.A. Stepanova, L.M. Tsybalova, O.I. Kiselev, N.V. Ravin, K.G. Skryabin, A molecular assembly system for presentation of antigens on the surface of HBc virus-like particles, Virology 435(2) (2013) 293-300.
[25] T. Tennikova, F. Svec, B. Belenkii, High-performance membrane chromatography. A novel method of protein separation, Journal of Liquid chromatography 13(1) (1990) 63-70.
[26] A. Oliva, M.a. Llabrés, J.B. Fariña, Comparative study of protein molecular weights by size-exclusion chromatography and laser-light scattering, Journal of pharmaceutical and biomedical analysis 25(5-6) (2001) 833-841.
[27] Y. Chen, Y. Zhang, C. Quan, J. Luo, Y. Yang, M. Yu, Y. Kong, G. Ma, Z. Su, Aggregation and antigenicity of virus like particle in salt solution—A case study with hepatitis B surface antigen, Vaccine 33 (2015).
[28] P. Vormittag, T. Klamp, J. Hubbuch, Ensembles of Hydrophobicity Scales as Potent Classifiers for Chimeric Virus-Like Particle Solubility - An Amino Acid Sequence-Based

Machine Learning Approach, Frontiers in bioengineering and biotechnology 8 (2020) 395-395.

Chapter 5 Immunogenicity evaluation of adjuvant-free chimeric Hepatitis B core (HBc) virus like particle (VLP) presenting Epstein-Barr nuclear antigens 1 (EBNA1) epitope

Abstract

Epstein-Barr virus (EBV) is regarded as the leading factor of infectious mononucleosis and is linked closely with several epithelial and lymphoid cancers. Since no licensed vaccine against EBV is developed, there is an urgent need for the development of an effective vaccine candidate for treatment of EBV related diseases and cancers. Epstein-Barr nuclear antigens 1 (EBNA1) is found to be crucial for the maintenance of EBV viral genome and its protein expression for survival. Here, virus-like particle (VLP) was applied to display EBNA1 epitope (aa: 407-417) for the potential vaccine development for protection of EBV infection. Hepatitis B virus core antigen (HBc) VLP was selected to carry and display short and non-structural EBNA1 epitope at the N-terminus. Chimeric EBNA1-HBc VLP was expressed in *Escherichia coli* (*E. coli)* expression system and purified by ammonium sulphate (AS) precipitation as described in **Chapter 3**. Immunogenicity evaluation results by animal test indicate that adjuvant free EBNA1-HBc VLP achieves similar level of epitope specific humoral and cellular

immune response compared with the groups with aluminium hydroxide adjuvant. The achieved immune response level is significantly higher than that achieved with only EBNA1 peptide. In addition, adjuvant free EBNA1-HBc VLP is proven to have long-term immunogenicity because of the evidence that vaccinated mice have produced epitope specific memory T cells in spleen. It suggests that HBc VLP platform is a promising candidate to present EBNA1 epitope and chimeric EBNA1-HBc is potentially an effective vaccine candidate for the protection of EBV infection with long-term protection.

Key words: Epstein-Barr virus; Virus-like particles; Hepatitis B virus core antigen (HBc); Adjuvant free; Long-term immunogenicity

5.1 Introduction

Epstein-Barr virus (EBV) is one of the γ-herpesvirus that is endemic in the world population and primarily infects B cells and epithelial cells[1]. Burkitt's lymphoma, Hodgkin lymphoma,[2], nasopharyngeal carcinoma, gastric and sporadic carcinoma tend to develop in EBV infected individuals[3, 4]. In addition, EBV has been proven to be related with the development of cancers such as lymphoid cancers[5], epithelial cancers[6] and pediatric leiomyosarcoma[7] by various factors[3]. Investigations have been conducted on the study of EBV, and it was found that EBV was able to transform cells due to the transactivating feature of Epstein-Barr nuclear antigens (EBNA)[8]. Therefore, EBNA have been studied as the antigens for the development of EBV vaccines[9-11].

In the absence of any commercial vaccines for the treatment of EBV, various vaccine candidates based on the capsid proteins of EBV were developed in the last decade against EBV infection[1, 9, 10, 12, 13]. However, the effectiveness, safety and long-term immunogenicity protection against EBV limited the application of these vaccine candidates. Epstein-Barr virus nuclear antigen-1 (EBNA1) protein was found in all tumours relating to EBV and it was regarded as a marker to identify the cancer cells caused by EBV. EBNA1 protein is crucial for the maintenance of EBV genome and viral DNA replication by binding with sequence-specific DNA[9]. EBNA1 has been reported to be related to cell transformation of EBV, however, the mechanism remains unclear[1, 14]. With these properties, EBNA1 protein is a promising antigen candidate

for the development of EBV vaccine.

Virus-like particles (VLPs) are promising candidates for the development of cancer vaccine delivery platform in the last decade[15]. Various VLPs from different viruses have been studied and evaluated, and now been adopted for the application for carrying foreign epitopes[16-18]. VLP-based vaccine platform possesses several benefits to develop safe and efficient cancer vaccines[19, 20]. Firstly, its repetitive antigenic structure ensures its good ability to present the foreign epitope in a high density and trigger high immune response[21, 22]. Secondly, it is a safer vaccine candidate compared with the traditional viral based vaccines because of its absence of the viral genome, which makes VLP-based vaccines non-infectious[23, 24]. Finally, VLP-based vaccines can be expressed and purified efficiently in the bacteria expression system with a relatively low cost[25, 26].

Taking advantages of the recent success of chimeric virus-like particle (VLP) vaccine strategies targeting hepatitis B virus (HBV) and human papillomavirus (HPV) and their associated tumours[27, 28], similar chimeric VLP-based vaccines for EBV related diseases and cancers could be developed. Among all VLP candidates, Hepatitis B virus core antigen (HBc) is the most widely used icosahedral VLPs carrier. These core particles are icosahedral nucleocapsids with primarily triangulation number T = 3 or T = 4 symmetry, each containing 180 and 240 units of 21 kDa core monomers[29]. In many ways, HBc VLPs attracts the researchers' attention because of its flexibility in the modification and construction. Moreover, HBc VLPs also show efficient self-

assembly ability in all known expression systems[30]. Several chimeric HBc VLP-based vaccines displaying foreign epitopes have been developed against viruses such as HCV[31], influenza virus[32] and foot-and-mouth disease virus (FMDV)[33].

To produce safe and effective vaccines is the ultimate goal to perform successful immunotherapy. To improve the immunogenicity, adjuvants are commonly used together with Active Pharmaceutical Ingredients (API) to form the vaccines. Aluminium adjuvant is one of the popular adjuvants and has been added in the commercial HBc VLP vaccines. However, reports have argued about the safety for the application of aluminium adjuvant as it may potentially cause post-immunization headache, arthralgia and myalgia[34]. In addition, reports have also indicated that aluminium adjuvant can improve the Th2 cells derived immune response with increased eosinophil and immunoglobulin (Ig) E production leading to the increase of the risk of allergy and anaphylaxis[35, 36].

In this study, the aim is to construct and evaluate the immunogenicity of a chimeric HBc VLP by presenting a short and non-structural antigen, EBNA1 epitope, by HBc VLP platform. Short EBNA1 peptide was designed to be fused to the N-terminus of HBc VLP to form the recombinant chimeric EBNA1-HBc VLP protein. Optimized expression and purification were conducted as described in **Chapter 3**. *In vivo* immune response of adjuvant free EBNA1-HBc VLP was evaluated compared with EBNA1-HBc VLP with aluminium adjuvant, HBc VLP and EBNA1 peptide in mice. Long-term protection ability of adjuvant free EBNA1-HBc VLP was evaluated by detection of

memory T cells produced in the immunized mice.

5.2 Materials and Methods

5.2.1 Expression and purification of EBNA1-HBc VLP

Chimeric EBNA1-HBc VLP was expressed and purified with the optimized condition as described in **Chapter 3**.

5.2.2 Immunization schemes

Female BALB/c mice aged at 6–8 weeks (body weight about 18–20 g) were purchased from SPF Biotechnology Co., Ltd. (Beijing, China) and maintained with pathogen-free water and food. The animals were randomly divided into 6 groups with 8 animals per group. The six groups were 1) control groups: PBS (negative control), Ovalbumin (OVA, positive control), EBNA1 peptide group, wt HBc VLP, 2) sample groups: EBNA1-HBc VLP without adjuvant, and EBNA1-HBc with aluminium hydroxide adjuvant (SERVA Electrophoresis GmbH, Germany) groups. For sample groups, the mice were immunized intraperitoneally with 100 μg of samples in 200 μl of sterile PBS on days 0, 14, and 28. For EBNA1 peptide group, 5.7 μg of EBNA1 peptide in 200 μl of sterile PBS was injected on days 0, 14, and 28. For HBc VLP group, 94 μg of HBc VLP in 200 μl of sterile PBS was injected on days 0, 14, and 28. For evaluation of the humoral immune response, immune serum was collected 10 days after first and second boost. For T-cell proliferation measurement and lymphocytes experiments, mice were sacrificed, and spleens were obtained at day 38 after the first immunization. Sera were

isolated and stored at -70 ℃.

5.2.3 ELISA for antibody titer

Enzyme-linked immunosorbent assay (ELISA) was performed for the detection of epitope specific antibody titer. Briefly, 100 μl of HBc VLP and EBNA1 peptide (aa 407-417) were coated overnight at 4 °C to 96-well plate at 10 μg/ml in 50 mM sodium carbonate buffer, pH 9.6. After being blocked with 200 μl of PBS containing 1 % bovine serum albumin (BSA) (Sigma-Aldrich, USA) for 2 h at 37°C, 100 μl of serial dilutions of sera samples from mice after immunized with EBNA1-HBc VLP with and without aluminium adjuvant for 24 and 38 days were added to the plate and incubated for an additional 2 h at 37°C. Same volume of serial diluted sera samples from mice after immunized with HBc VLP group and EBNA1 peptide group were used as references. After being washed three times with PBS containing 0.1 % Tween-20, 100 μl of anti-mouse IgG conjugated with horseradish peroxidase (Solarbio Life Sciences, China) was added at a 1:5,000 dilution. Following 2 h of incubation at 37°C, the plate was washed, and 100 μl of 3,3′,5,5′-Tetramethylbenzidine (TMB) single-component substrate solution (Sigma-Aldrich, USA) was added for colour development. The colour development was stopped by adding 100 μl of 1 M H_2SO_4. The results were recorded in a microplate reader (Perlong, China) at wavelength of 450 nm. The endpoint titers were defined as the highest serum dilution that resulted in an absorbance value two times more than that of negative-control sera derived from nonimmunized mice.

IgG isotype was also examined using similar ELISA process as described above to

evaluate the Th1/Th2 tendency of immune response triggered by produced chimeric EBNA1-HBc VLP. Briefly, 100 µl of EBNA1 peptide (aa 407-417) was coated overnight at 4°C to 96-well plate at 10 µg/ml in 50 mM sodium carbonate buffer, pH 9.6 and the plate was divided into two parts. The plate was then blocked with 200 µl of PBS containing 1 % bovine serum albumin (BSA) (Sigma-Aldrich, USA) for 2 h at 37°C. Sera samples after immunized with EBNA1-HBc VLP with and without aluminium adjuvant for 38 days were diluted with same ratio at the endpoint. 100 µl of diluted sera samples from both groups were added to. After being washed three times with PBS containing 0.1 % Tween-20, 100 µl of goat anti-mouse IgG1 and goat anti-mouse IgG2a (Sigma-Aldrich, USA) were added to the two parts of the plate, respectively at a dilution rate of 1:1,000. The plate was washed three times with PBS containing 0.1 % Tween-20. After washing, 100 µl of rabbit anti-goat IgG conjugated with horseradish peroxidase (Beijing Biosynthesis Biotechnology Co., Ltd., China) was added at a 1:1,000 dilution. Following 2 h of incubation at 37°C, the plate was washed, and 100 µl of TMB single-component substrate solution (Sigma-Aldrich, USA) was added for colour development. The colour development was stopped by adding 100 µl of 1 M H_2SO_4. The results were checked in a microplate reader (Perlong, China) at wavelength of 450 nm.

5.2.4 T-cell proliferation assay

Murine splenocytes were harvested using red blood cell lysing buffer (Sigma-Aldrich, USA). Single-cell suspensions were prepared (4×10^6 cells/ml) and cultured in RPMI

1640 (Gibco, Germany) with the appropriate HBc VLP and EBNA1 peptide at 20 μg/ml. Concanavalin A (Sigma-Aldrich, USA) was used as a positive control at 2 μg/ml. The plate was then incubated for 48 h before the addition of 10 μl of Cell Counting Kit – 8 (CCK-8) (Sigma-Aldrich, USA) per well. The absorbance at wavelength of 450 nm was recorded by microplate reader (Perlong, China) after 4 h incubation at 37°C. The more and faster the cells proliferate, the higher the detected absorbance would be. The results were presented as proliferation indexes (PI) using the following equation.

$$Proliferation\ rate = \frac{A(sample) - A_0(control)}{A_0(control)}$$ ----------(**Eq. 5.1**)

A(sample): A450 nm of the sample group with CCK-8 and stimulated with EBNA1 peptide

A_0(control): A450 nm of the control group with CCK-8 only

$$Proliferation\ Index = \frac{Proliferation\ rate\ (sample)}{Proliferation\ rate\ (PBS)}$$ ----------(**Eq. 5.2**)

5.2.5 Cytokine test

For detection of gamma interferon (IFN-γ), interleukin 2 (IL-2), interleukin 4 (IL-4), and interleukin 10 (IL-10) cytokines in cell cultures, the spleen cells from the mice immunized with EBNA1-HBc VLP with and without aluminium adjuvant were seeded in the 24 well plate at 4 x 10^6 cells/ml. After culturing for 24 and 48 hours, cells and culture media were collected and centrifuged at 1000 rpm for 10 min. The supernatants were used for cytokine assay. Analysis for the presence of cytokines in cell supernatants was performed using the commercial tests OptEIA Mouse IFN-γ, IL-2, IL-4 and IL-10 ELISA Sets (BD Biosciences, USA) according to the manufacturers' instructions.

Briefly, 96 well plate was coated with 100 µL/well of capture antibody in PBS buffer (supplied in the kit). The plate was sealed and incubated overnight at 4°C. Plate was washed 3 times with PBS buffer with 0.05 % Tween-20. Wells were blocked with 200 µL of ELISA/ELISPOT Diluent (1X) and incubated at room temperature for 1 hour. After washing with PBS buffer with 0.05 % Tween-20, 2-fold serial dilutions of the standards (provided in the kit) to make the standard curve for a total of 8 points. 100 µL/well of supernatant samples of lymphocyte culture were then added to the appropriate wells. 100 µL of ELISA/ELISPOT Diluent (1X) was added to the blank well and the plate was sealed and incubated at room temperature for 2 hours. After washing for five times with PBS buffer with 0.05 % Tween-20, 100 µL/well diluted Detection Antibody (provided in the kit) was added to all wells. The plate was then sealed and incubated at room temperature for another 1 hour. 100 µL/well of diluted Streptavidin-HRP (provided in the kit) was added after washing plate for five times with PBS buffer with 0.05 % Tween-20 and incubate at room temperature for 30 min. 100 µL/well of 1X TMB Solution was added after five times washing with PBS buffer with 0.05 % Tween-20 and incubate at room temperature for 15 minutes for colourisation. Finally, 100 µL/well of 1 M H2SO4 was added to each well stop the reaction. The absorbance at wavelength of 450 nm was recorded in a microplate reader (Perlong, China).

5.2.6 Lymphocyte activation

Splenocytes from immunized mice with and without EBNA1 peptide were cultured for

60 h at 37°C in 24 well plate with cell density of 4×10^6 cells/ml before mixing with FITC CD4 Monoclonal antibody, PerCP-Cy 5.5 CD8a Monoclonal antibody, APC-eFluor 780 CD19 Monoclonal antibody and APC CD25 Monoclonal antibody (Thermo fisher, USA) at 4 °C. After washing with PBS, fluorescence patterns were analysed using a FACSCanto II (BD Biosciences, USA). The percentage of $CD25^+/CD4^+$ T cells, $CD25^+/CD8^+$ T cells and $CD25^+/CD19^+$ B cells were recorded and analysed using FACSCanto II software.

5.2.7 Memory T cells activation assay

Similar to lymphocyte activation assay, splenocytes from immunized mice with and without EBNA1 peptide were cultured for 60 h at 37°C in 24 well plate with cell density of 4 x 10^6 cells/ml before mixing with FITC CD4 Monoclonal antibody, PerCP-Cy 5.5 CD8a Monoclonal antibody, PE CD62L (L-Selectin) Monoclonal Antibody and PE-Cyanine7 CD44 Monoclonal Antibody, (Thermo fisher, USA) at 4 °C. After washing with PBS, fluorescence patterns were analysed using a FACSCanto II (BD Biosciences, USA). The percentage of $CD44^{hi}CD62L^{hi}$/CD4+ T cells, $CD44^{hi}CD62L^{low}$/CD4+, $CD44^{hi}CD62L^{hi}$ /CD8+ T cells and $CD44^{hi}CD62L^{low}$/CD8+ T cells were recorded and analysed using FACSCanto II software.

5.2.8 Statistical analysis

Statistical results were analysed using GraphPad-Prism 6 software (GraphPad Software, USA). The values of $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were considered statistically significant between the experimental groups, respectively.

5.2.9 Ethical statement

This study was approved by the Animal Ethics Committee of Shanxi University of Chinese Medicine (Shanxi, China Approval number: 2019LL137).

5.3 Results

5.3.1 Humoral response of EBNA1-HBc VLP

Serum of mice was collected on day 24 and 38 after initial vaccination. The 2nd boost resulted in a substantial improvement of EBNA1 specific antibody response (**Figure 5.1**). Mice immunized with wt HBc VLP and EBN1 peptide were applied as reference groups. As shown in **Figure 5.1**, anti-EBNA1 specific antibody titers achieved after 2nd boost vaccinated with chimeric EBNA1-HBc VLP with and without adjuvant were significantly higher than that after 1st boost. The anti-EBNA1 specific antibody titer of EBNA1-HBc VLPs with and without adjuvant reached 2.7×10^5 and 3.1×10^5 after 2nd boost, respectively. The EBNA1 specific humoral immune response vaccinated with chimeric EBNA1-HBc VLP group improved significantly compared with the group vaccinated with only EBNA1 peptide where the anti-EBNA1 antibody titer was 8.9×10^3 after 2nd boost. The anti-EBNA1 specific antibody titer of EBNA1-HBc VLP group with adjuvant showed only a slight improvement compared with that of adjuvant free group. In addition, the insertion of the EBNA1 epitope to the HBc VLP caused a dramatic reduction of HBc VLP specific response where the antibody titer was around 4×10^5. The reduced carrier specific immune response could be one merit for the chimeric HBc VLP vaccines presenting foreign epitopes since the carrier specific

immune response is not the effective response. However, the total antibody titer value achieved by chimeric EBNA1-HBc VLP groups was found to be lower compared with total antibody titer of wt HBc VLP group. This could be resulted from the carrier induced epitopic suppression (CIES) effect[37].

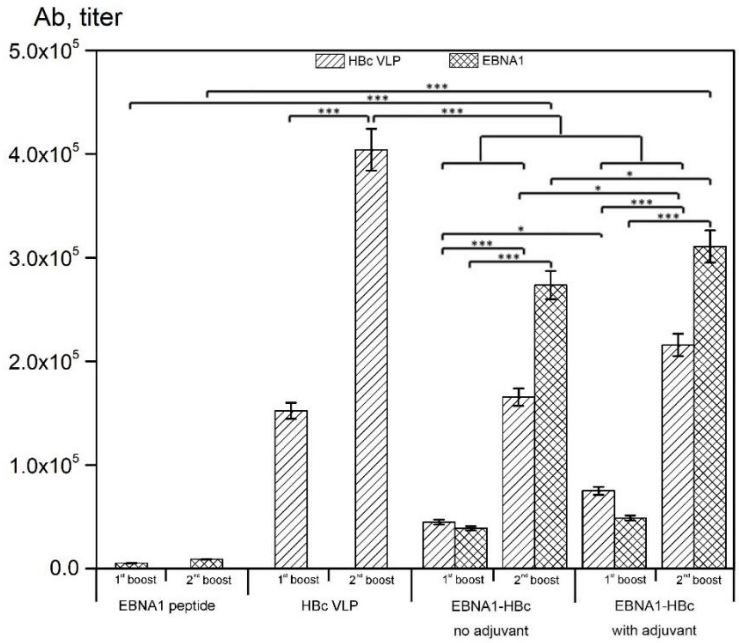

Figure 5.1 Induction of antibodies (Ab) in mice by chimeric HBc-derived VLPs. Shown are antibody titers in pools of sera from mice at day 24 (1st boost) and at day 38 (2nd boost) after the first immunization. Titers in specimens were determined on plate coated with HBc VLP and EBNA1 peptide. Antibody titers of mice treated with EBNA1 peptide and HBc VLP were used as references. Data are expressed as mean ± SD (n=8) (*p<0.05; **p<0.01; ***p<0.001).

5.3.2 Cellular immune response and cytokine assay

The proliferations of T-cells of mice vaccinated with chimeric EBNA1-HBc VLP with and without adjuvant were examined to demonstrate the cellular immune response level after stimulated with EBNA1 peptide and HBc VLP *in vitro* at day 38 after the first immunization. As shown in **Figure 5.2**, T-cell proliferations of both chimeric EBNA1-HBc VLP groups with and without adjuvant were detected against EBNA1 peptide. EBNA1 peptide specific proliferation *in vitro* of chimeric EBNA1-HBc VLPs with and

without adjuvant groups both improved more than three folds compared with the group vaccinated with EBNA1 peptide. However, the total proliferation index of these two groups were significantly lower compared with HBc VLP groups because of the CIES effect[37]. With the addition of adjuvant, the proliferation index of EBNA1-HBc VLP with adjuvant group was slightly higher than that of adjuvant free group. **Figure 5.3** illustrates the direct evaluation of the Th1 cell-derived cytokines IFN-γ, IL-2, and IL-10 after stimulation with EBNA1 peptide and HBc VLP with 24 h and 48 h. Culture of 48 h showed significantly higher levels of all cytokines and it is possible because more lymphocytes were proliferated and activated. Among Th1 cell derived cytokines, production of IL-2 was relatively reduced compared with production of IFN-γ and IL-10. Produced IFN-γ by adjuvant free EBNA1-HBc VLP and EBNA1-HBc VLP with adjuvant reached 63.6 pg/ml and 73.1 pg/ml, respectively, after being stimulated by EBNA1 peptide for 48 h and produced IL-10 reached 570 pg/ml and 515 pg/ml, respectively while produced IL-2 only reached 17.3 pg/ml and 26 pg.ml, respectively. Th2 cell-derived cytokine, IL-4 was also examined. The production of IL-4 was still detectable; however, the production of IL-4 was lower than all Th1 cell derived cytokines. The produced IL-4 by adjuvant free EBNA1-HBc VLP and EBNA1-HBc VLP with adjuvant were 19.1 pg/ml and 21.3 pg/ml, respectively, after being stimulated by EBNA1 peptide for 48 h. In addition, only slightly difference was detected between the produced cytokines by adjuvant free EBNA1-HBc VLP and the group with aluminium adjuvant after being stimulating by EBNA1 peptide.

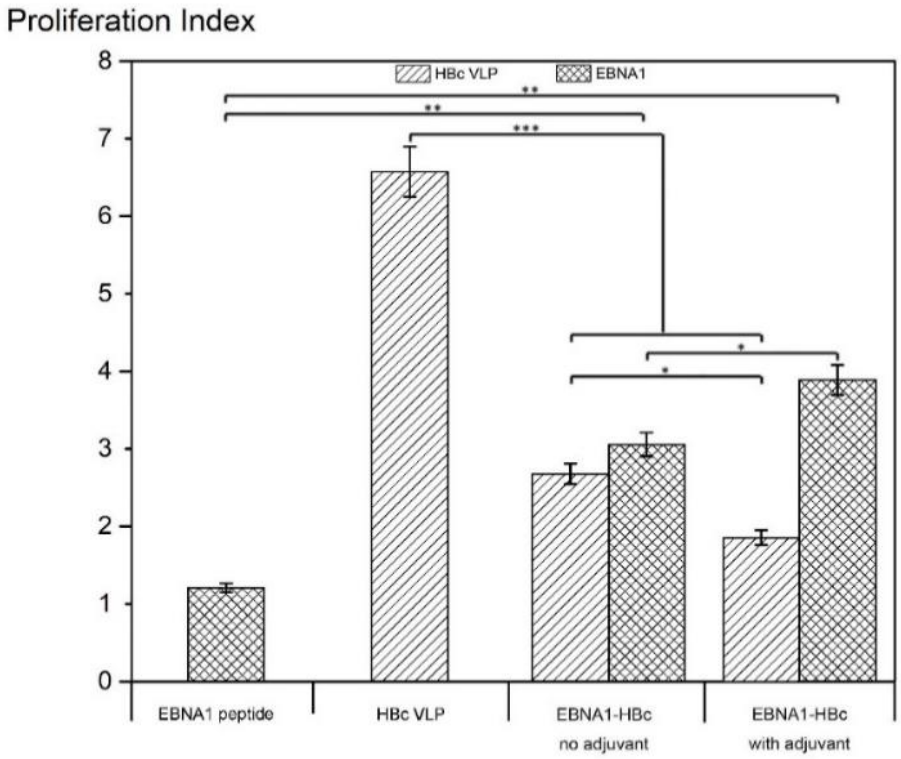

Figure 5.2 T-cell proliferation after immunization of mice by EBNA1-HBc VLPs, wt HBc VLP and EBNA1 peptide. PI were measured in response to stimulation of T cells with HBc VLP and EBNA1 peptide. The error bars indicate standard deviations. Proliferation results for T-cells of mice treated with EBNA1 peptide and HBc VLP were used as references. Data are expressed as mean ± SD (n=8) (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

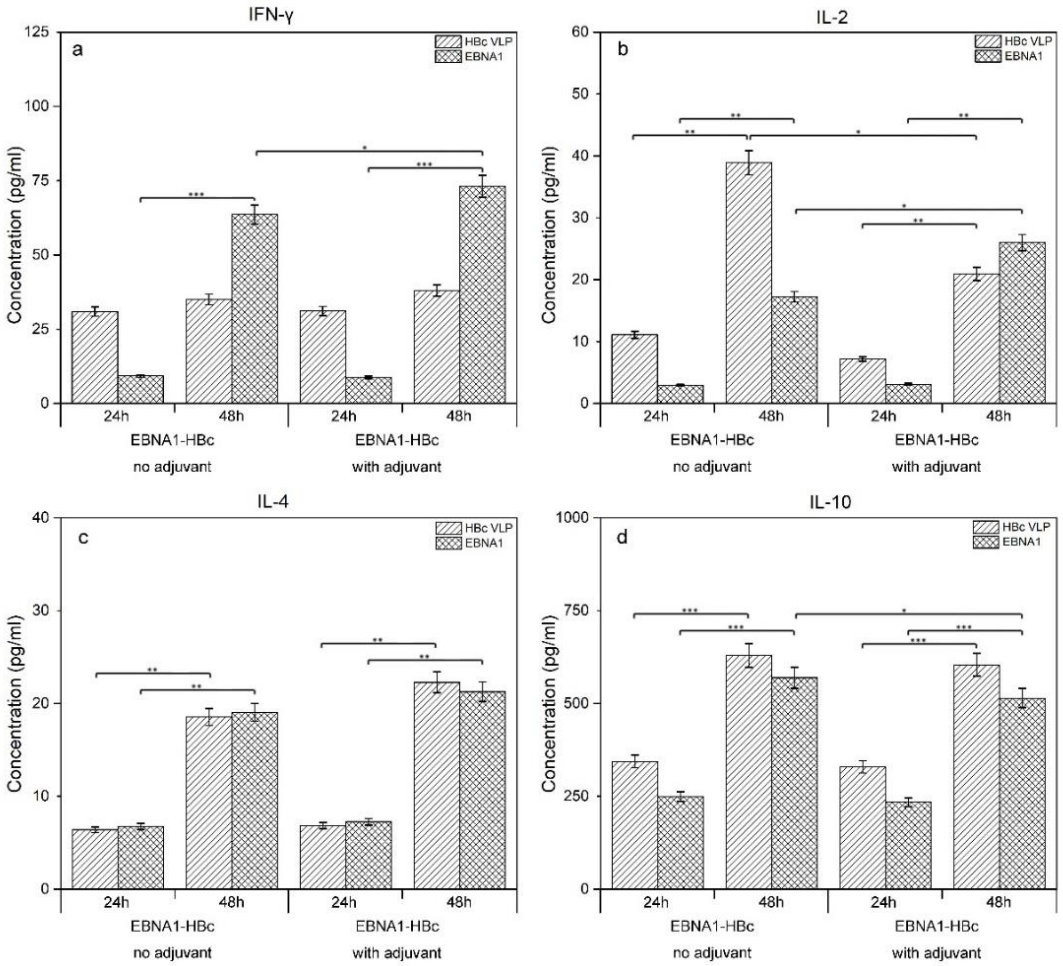

Figure 5.3 Cytokines produced by T cells after immunization of mice with EBNA1-HBc VLPs. Cell supernatants were removed and analysed at 24 h and 48 h for IFN-γ(a), IL-2 (b), IL 4 (c) and IL-10 (d) production after stimulation with HBc VLP and EBNA1 peptide. Data are expressed as mean ± SD (n=8) (*p<0.05; **p<0.01; ***p<0.001).

5.3.3 IgG isotype of chimeric EBNA1-HBc VLP

Specific isotypes of antibodies of mice vaccinated with EBNA1-HBc VLP with and without aluminium adjuvant were analysed and shown in **Figure 5.4**. Both groups of EBNA1-HBc VLP with and without adjuvant showed a predominantly EBNA1 specific IgG1 antibody response, indicating that EBNA1-HBc VLP has the tendency of Th2 cells derived immune response. In addition, a slightly improved tendency of Th2 cells derived immune response was observed in EBNA1-HBc VLP group after addition of

aluminium adjuvant.

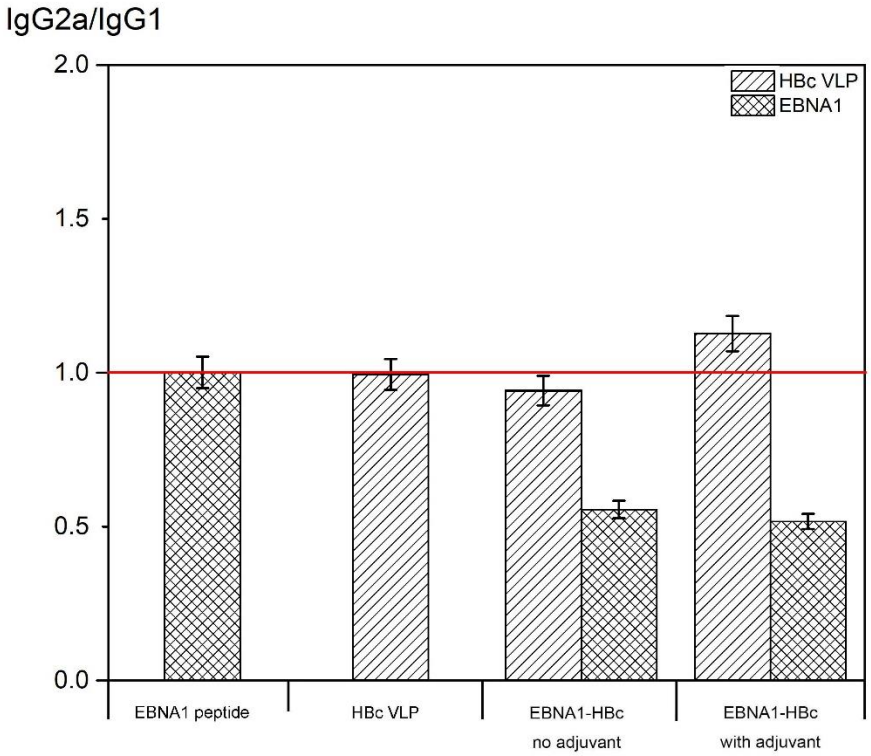

Figure 5.4 IgG isotype distribution in serum collected 10 days after 2nd boost vaccination of mice by chimeric EBNA1-HBc VLP with and without adjuvant. EBNA1 peptide and wt HBc VLP groups were used as references. The red line depicts the equivalent distribution of IgG2a and IgG1 isotypes.

5.3.4 Lymphocyte activation

CD25 marker is the activation marker for T cells and B cells, indicating the activation level of lymphocytes after being stimulated with specific antigens. To further review the immune response of chimeric EBNA1-HBc VLP, the percentage of activated lymphocytes was evaluated in $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells using the flow cytometry (**Figure 5.6**). As shown in **Figure 5.5**, both chimeric EBNA1-HBc VLP with and without adjuvant groups showed a significant increase in the percentage of activated $CD4^+$ T cells and $CD19^+$ B cells. The percentage of $CD4^+$ T cells and $CD19^+$ B cells induced by adjuvant free EBNA1-HBc increased from 0.9 % and 1 % to 2.6 %

and 3.7 %, respectively. However, the increase of the activated $CD8^+$ T cells was minor, suggesting that the cellular immune response triggered by the adjuvant free EBNA1-HBc VLP is potentially lower than the humoral immune response. After adding the adjuvant, the cellular immune response level improved slightly compared with the one without adjuvant. The results suggest that when displaying EBNA1 epitope on the HBc VLP platform, a stronger humoral immune response than the cellular immune response would be achieved, and this explained high level of EBNA1-specific antibody titer.

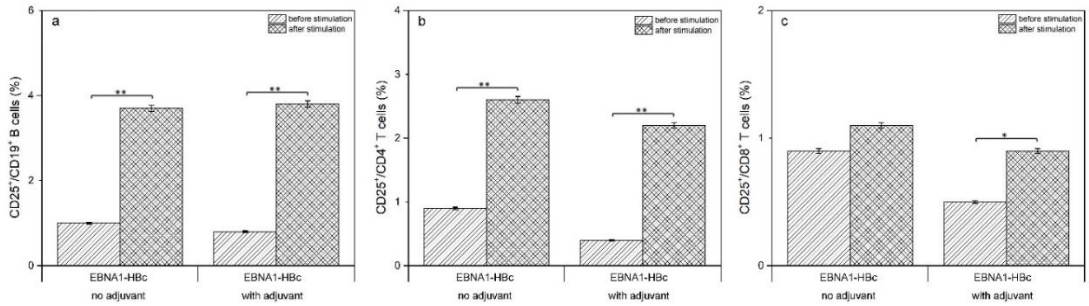

Figure 5.5 Activation of $CD19^+$ B cells (a), $CD4^+$ T cells (b) and $CD8^+$ T cells (c) of lymphocytes after immunization of mice with EBNA1-HBc VLPs stimulated by EBNA1 peptide. Data are expressed as mean ± SD (n=3) (*p<0.05; **p<0.01; ***p<0.001).

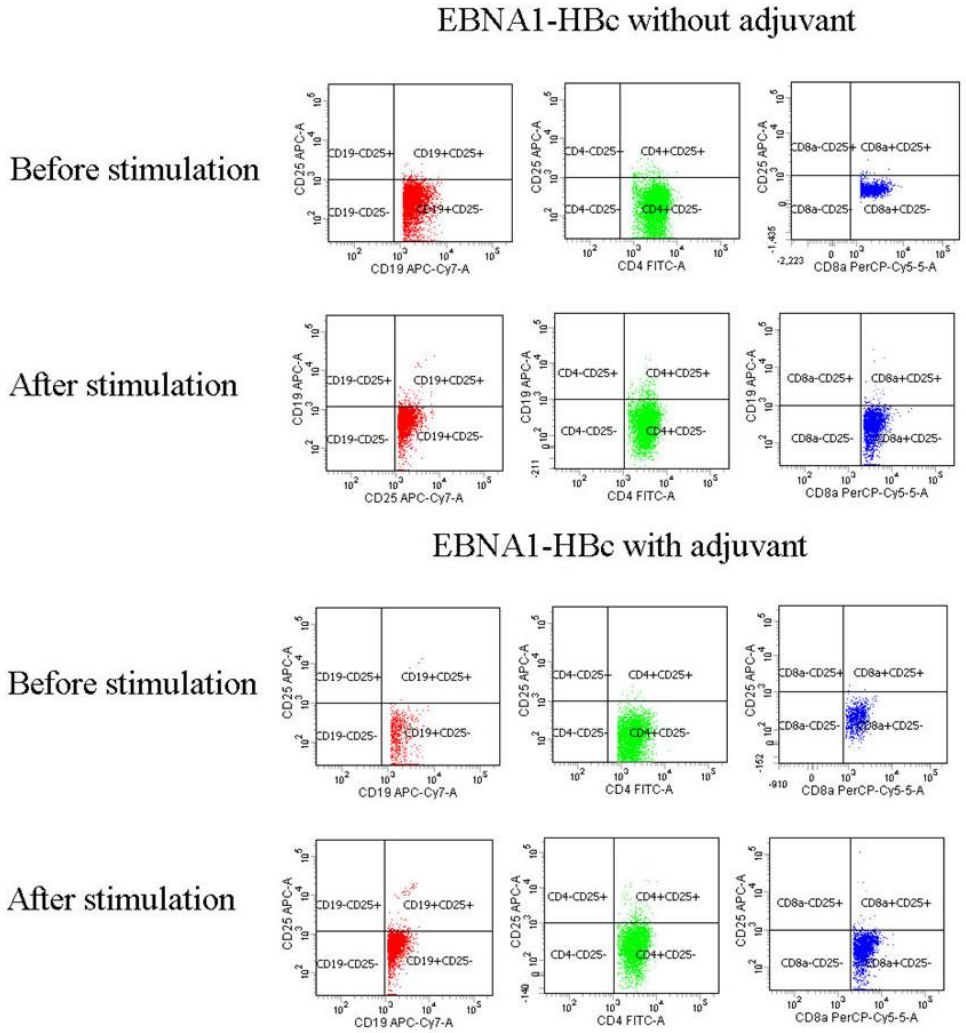

Figure 5.6 Flow cytometry of CD25 marker in $CD19^+$ B cells, $CD4^+$ T cells and $CD8^+$ T cells of lymphocytes after immunization of mice with EBNA1-HBc VLPs stimulated by EBNA1 peptide.

5.3.5 Memory T cells

Long-term protection is the primary feature for a successful vaccine development. To evaluate the long-term protection property of constructed vaccine samples, the percentage of memory $CD4^+$ and $CD8^+$ T cells were evaluated. CD44 and CD62L are two markers on the surface of memory T cells. On the surface of central memory T cells (T_{CM} cells), CD44 and CD62L makers are highly expressed, while on the surface of effector memory T cells (T_{EM} cells), CD44 maker is highly expressed and CD62L is

expressed in low level. The surface markers of $CD4^+$ and $CD8^+$ T cells of the mice vaccinated with chimeric EBNA1-HBc VLP were evaluated with flowcytometry (**Figure 5.8**). As shown in **Figure 5.7**, a significant decrease in the percentage of $CD4^+$ and $CD8^+$ T_{CM} cells was detected after vaccinated with EBNA1-HBc VLP with and without adjuvant. The percentage of $CD4^+$ and $CD8^+$ T_{EM} cells were increased after being stimulated with specific epitope. The percentage of $CD4^+$ and $CD8^+$ T_{CM} cells induced by adjuvant free EBNA1-HBc VLP decreased from 2 % and 3.5 % to 0.9 % and 1.1 %, respectively, and the percentage of $CD4^+$ and $CD8^+$ T_{EM} cells increased from 12.8 % and 15.8 % to 26.5 % and 22 %, respectively. This demonstrates the successful production of the central memory T cells targeting to specific epitope after immunized with produced EBNA1-HBc VLP.

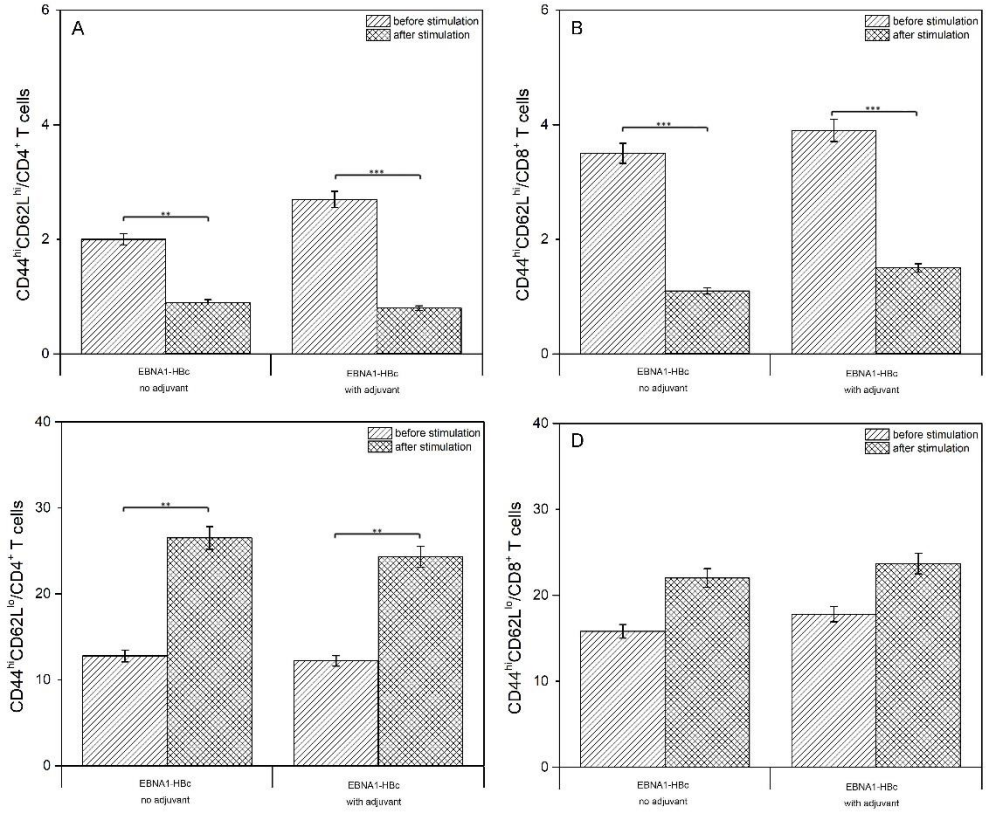

Figure 5.7 Measurement of central ($CD44^{hi}CD62L^{hi}$)/effector ($CD44^{hi}CD62L^{low}$) memory CD4+ and CD8+ T cells. Splenocytes were harvested on day 38 after the first immunization and stimulated *in vitro* by EBNA1 peptide for 60 h. The frequency of $CD44^{hi}CD62L^{hi}/CD4^{+}$ T cells, $CD44^{hi}CD62L^{low}/CD4^{+}$ T cells, $CD44^{hi}CD62L^{hi}/CD8^{+}$ T cells, and $CD44^{hi}CD62L^{low}/CD8^{+}$ T cells were measured by flow cytometry. Data are expressed as mean ± SD (n=3) (*p<0.05; **p<0.01; ***p<0.001).

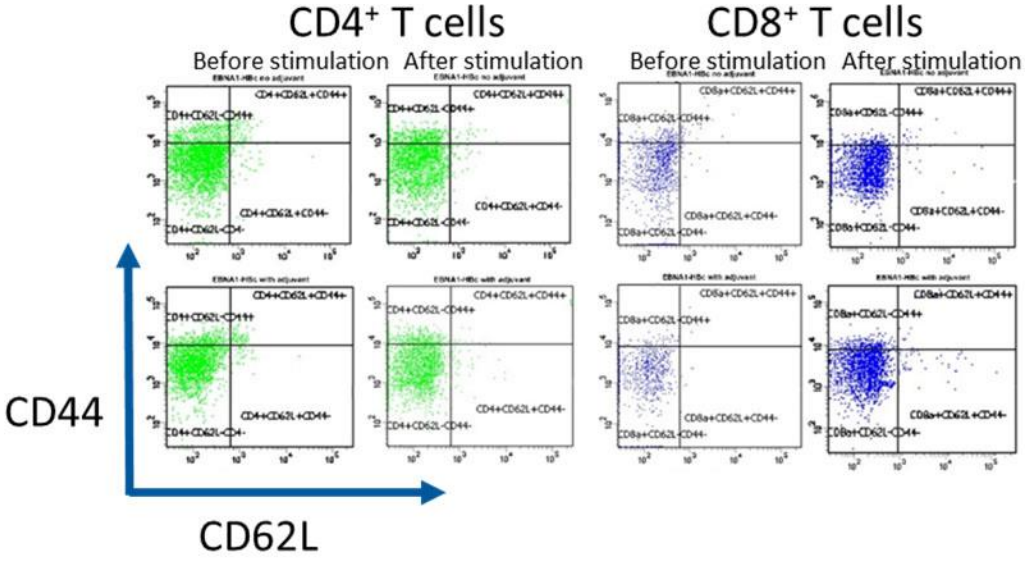

Figure 5.8 Flow cytometry of central /effector memory $CD4^{+}$ and $CD8^{+}$ T cells.

5.4 Discussion

In vivo immunogenicity of EBNA1-HBc VLP with and without addition of aluminium adjuvant were evaluated through various experiments. Both EBNA1-HBc groups with and without adjuvant elicit significantly higher EBNA1 specific humoral immune response compared with the group immunized with only EBNA1 peptide. In addition, the epitope specific antibody titer level achieved by adjuvant free EBNA1-HBc VLP reached 10^5 and this is higher or similar to other reported EBV vaccine candidates such as chimeric EBVgp350/220 VLP produced by Ogembo's research group with the antibody titer level of 10^2 without adjuvant and chimeric gp350-HBc VLP produced by Zhang's group with the antibody titer level of 10^5 with addition of aluminium adjuvant[38, 39]. When analysing the influence of adjuvant on the immune response, adjuvant free EBNA1-HBc VLP induced a similar immune response in mice compared with the group with aluminium adjuvant. This may be because of the self-adjuvant property of HBc VLP[40]. The cellular immune response results also indicate that adjuvant free EBNA1-HBc VLP induced three times higher cellular immune response compared with EBNA1 peptide and no significant difference was found between adjuvant free group and the group with aluminium adjuvant. In addition to examination of the immune response level of adjuvant free EBNA1-HBc VLP, predominant immune response type was also examined by evaluating the IgG isotype ratio. As a result, both EBNA1-HBc VLP with and without aluminium adjuvant elicited predominant humoral immune response. The group with aluminium adjuvant shows a slightly larger tendency

for humoral immune response because of the propensity of aluminium adjuvant to induce IL-1[41]. These results prove that adjuvant free EBNA1-HBc VLP is able to be applied as an effective vaccine candidate to trigger strong epitope specific humoral and cellular immune response.

To further evaluate the immune response after being stimulated with EBNA1 peptide, the activation of T cells and B cells was examined for EBNA1-HBc VLP with and without adjuvant groups. CD25 maker was used to prove the successful activation of T cells and B cells. CD 25 is the alpha chain of the trimeric IL-2 receptor and is commonly used as the marker to detect the activation of T cells and B cells[42, 43]. Both EBNA1-HBc VLP groups with and without aluminium adjuvant demonstrate a significant increase of the percentage of activated $CD4^+$ cells and $CD19^+$ B cells after stimulation with EBNA1 peptide where the percentage of $CD4^+$ cells and $CD19^+$ B cells increased by 1.7 % and 2.7 %, respectively, while the percentage of activated $CD8^+$ T cells increased slightly. This is for the reason that EBNA1-HBc predominately induces Th2 derived humoral immune response as discussed previously.

In addition to the evaluation of immunogenicity and activation of lymphocytes, EBNA1 specific long-term immunogenicity was detected by measuring the central memory T cells. Long term protection is regarded as the ultimate goal for a successful vaccination[44]. There are two types of memory T cell that are responsible for long-term immunogenicity, central memory T cells (T_{CM} cells) and effective memory T cells (T_{EM} cells). T_{CM} cells have a longer lifetime but the respond is slower, while T_{EM} cells

have a shorter lifetime but perform rapid respond[45]. It is also reported that after stimulation with antigen, T_{CM} cells could differentiate into T_{EM} cells[46]. On the surface of T_{CM} cells, CD44 and CD62L makers are highly expressed, while on the surface of T_{EM} cells, CD44 maker is highly expressed and CD62L is expressed in low level. In this case, CD44 and CD62L marker were detected in our study to evaluate the density of memory T cells among lymphocytes in the spleen of immunized mice. EBNA1 peptide was used as the stimulator to examine the specific response of memory T cells against EBNA1 peptide. Results indicate that mice immunized with both adjuvant free EBNA1-HBc VLP group and EBNA1-HBc VLP with aluminium adjuvant group are able to produce $CD4^+$ T_{CM} cells, $CD4^+$ T_{EM} cells, $CD8^+$ T_{CM} cells and $CD8^+$ T_{EM} cells. After being stimulated with EBNA1 peptide, the percentage of $CD4^+$ T_{CM} cells and $CD8^+$ T_{CM} cells of both adjuvant free EBNA1-HBc VLP and EBNA1-HBc with aluminium adjuvant group decreased significantly. This confirms that the produced T_{CM} cells have a specific response against EBNA1 peptide and also potentially demonstrates the long-term protection ability of EBNA1-HBc VLP. No significant difference was found between adjuvant free EBNA1-HBc VLP group and EBNA1-HBc VLP with aluminium adjuvant group. In addition, after stimulation with EBNA1 peptide, the percentage of $CD4^+$ T_{EM} cells increased more than the increase of the percentage of $CD8^+$ T_{EM} cells. This matches with our previous finding that EBNA1-HBc VLP elicits predominately Th2 cell derived humoral immune response against EBNA1 peptide.

5.5 Conclusion

In our study, EBNA1 epitope is successfully presented using HBc VLP platform for the first time. The chimeric EBNA1-HBc was expressed and purified according to previous work in **Chapter 3**. *In vivo* immunogenicity evaluation demonstrates that adjuvant free EBNA1-HBc VLP is able to elicit strong EBNA1 specific humoral and cellular immune response due to its self-adjuvant property with the antibody titer level of 10^5 and three folds of T-cell proliferation compared with EBNA1 peptide group. This property potentially ensures that adjuvant free EBNA1-HBc VLP is able to be applied as an effective vaccine candidate for the protection of EBV infection. In addition, adjuvant free EBNA1-HBc VLP also shows long-term protection property as the mice vaccinated with EBNA1-HBc VLP can successfully produce epitope specific memory T cells and the activation of these memory T cells after stimulation with EBNA1 peptide is confirmed. These findings demonstrate that HBc VLP platform is promising to present short and non-structural antigen like EBNA1 epitope as an adjuvant free vaccine candidate to elicit epitope specific strong and long-lasting immune response. However, further EBV neutralization assay is needed to confirm that adjuvant free EBNA1-HBc VLP is able to be used as a vaccine candidate for the treatment of EBV infection.

Reference

[1] P.J. Farrell, Epstein–Barr Virus and Cancer, Annual Review of Pathology: Mechanisms of Disease 14(1) (2019) 29-53.

[2] A.M. Price, M.A. Luftig, Chapter Six - Dynamic Epstein–Barr Virus Gene Expression on the Path to B-Cell Transformation, in: K. Maramorosch, F.A. Murphy (Eds.), Adv. Virus Res., Academic Press2014, pp. 279-313.

[3] H. Hjalgrim, J. Friborg, M. Melbye, The epidemiology of EBV and its association with malignant disease, in: A. Arvin, G. Campadelli-Fiume, E. Mocarski, P.S. Moore, B. Roizman, R. Whitley, K. Yamanishi (Eds.), Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis, Cambridge University Press

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[4] M.P. Thompson, R. Kurzrock, Epstein-Barr Virus and Cancer, Clinical Cancer Research 10(3) (2004) 803.

[5] C. Shannon-Lowe, A.B. Rickinson, A.I. Bell, Epstein-Barr virus-associated lymphomas, Philosophical transactions of the Royal Society of London. Series B, Biological sciences 372(1732) (2017) 20160271.

[6] R. Ayee, M.E.O. Ofori, E. Wright, O. Quaye, Epstein Barr Virus Associated Lymphomas and Epithelia Cancers in Humans, J. Cancer 11(7) (2020) 1737-1750.

[7] M.T. Tetzlaff, C. Nosek, C.L. Kovarik, Epstein-Barr virus-associated leiomyosarcoma with cutaneous involvement in an African child with human immunodeficiency virus: a case report and review of the literature, J. Cutan. Pathol. 38(9) (2011) 731-9.

[8] G. Singh, S. Aras, A.H. Zea, S. Koochekpour, A. Aiyar, Optimal Transactivation by Epstein-Barr Nuclear Antigen 1 Requires the UR1 and ATH1 Domains, J. Virol. 83(9) (2009) 4227-4235.

[9] J.B. Wilson, E. Manet, H. Gruffat, P. Busson, M. Blondel, R. Fahraeus, EBNA1: Oncogenic Activity, Immune Evasion and Biochemical Functions Provide Targets for Novel Therapeutic Strategies against Epstein-Barr Virus- Associated Cancers, Cancers (Basel) 10(4) (2018) 109.

[10] C. Gurer, T. Strowig, F. Brilot, M. Pack, C. Trumpfheller, F. Arrey, C.G. Park, R.M. Steinman, C. Münz, Targeting the nuclear antigen 1 of Epstein-Barr virus to the human endocytic receptor DEC-205 stimulates protective T-cell responses, Blood 112(4) (2008) 1231-1239.

[11] N. Blake, T. Haigh, G. Shaka'a, D. Croom-Carter, A. Rickinson, The Importance of Exogenous Antigen in Priming the Human CD8+ T Cell Response: Lessons from the EBV Nuclear Antigen EBNA1, The Journal of Immunology 165(12) (2000) 7078-7087.

[12] D.G. van Zyl, J. Mautner, H.-J. Delecluse, Progress in EBV Vaccines, Front. Oncol. 9(104) (2019).

[13] R. Ruiss, S. Jochum, G. Wanner, G. Reisbach, W. Hammerschmidt, R. Zeidler, A Virus-Like Particle-Based Epstein-Barr Virus Vaccine, J. Virol. 85(24) (2011) 13105-13113.

[14] J.B. Wilson, E. Manet, H. Gruffat, P. Busson, M. Blondel, R. Fahraeus, EBNA1: Oncogenic Activity, Immune Evasion and Biochemical Functions Provide Targets for

Novel Therapeutic Strategies against Epstein-Barr Virus- Associated Cancers, Cancers 10(4) (2018).

[15] N.K.C. Linda H.L. Lua, Frank Sainsbury, Yap P. Chuan,Nani Wibowo, Anton P.J. Middelberg, Bioengineering Virus-Like Particles as Vaccines, Biotechnology and Bioengineering 111 (2014) 425-440.

[16] M.O. Mohsen, D.E. Speiser, A. Knuth, M.F. Bachmann, Virus-like particles for vaccination against cancer, Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology n/a(n/a) e1579.

[17] D. Yan, Y.-Q. Wei, H.-C. Guo, S. Sun, The application of virus-like particles as vaccines and biological vehicles, Applied microbiology and biotechnology 99 (2015).

[18] H.K. Ong, W.S. Tan, K.L. Ho, Virus like particles as a platform for cancer vaccine development, PeerJ 5 (2017) 4053-4053.

[19] C. Lemke, A. Salem, A. Krieg, G. Weiner, Abstract 1417: Combination cancer immunotherapy using checkpoint blockade and intratumoral virus-like particles containing CpG ODN, Cancer Res. 76(14 Supplement) (2016) 1417.

[20] L. Klimek, T. Kündig, M.F. Kramer, S. Guethoff, E. Jensen-Jarolim, C.B. Schmidt-Weber, O. Palomares, M.O. Mohsen, T. Jakob, M. Bachmann, Virus-like particles (VLP) in prophylaxis and immunotherapy of allergic diseases, Allergo Journal International 27(8) (2018) 245-255.

[21] S.A. Keller, M. Bauer, V. Manolova, S. Muntwiler, P. Saudan, M.F. Bachmann, Cutting Edge: Limited Specialization of Dendritic Cell Subsets for MHC Class II-Associated Presentation of Viral Particles, The Journal of Immunology 184(1) (2010) 26.

[22] A.F. Sander, P.-L. Lollini, Virus-like antigen display for cancer vaccine development, what is the potential?, Expert Review of Vaccines 17(4) (2018) 285-288.

[23] J. Chroboczek, I. Szurgot, E. Szolajska, Virus-like particles as vaccine, Acta Biochim Pol 61(3) (2014) 531-9.

[24] R. Noad, P. Roy, Virus-like particles as immunogens, Trends Microbiol. 11(9) (2003) 438-44.

[25] A.P. Middelberg, T. Rivera-Hernandez, N. Wibowo, L.H. Lua, Y. Fan, G. Magor, C. Chang, Y.P. Chuan, M.F. Good, M.R. Batzloff, A microbial platform for rapid and low-cost virus-like particle and capsomere vaccines, Vaccine 29(41) (2011) 7154-62.

[26] M.W. Liew, A. Rajendran, A.P. Middelberg, Microbial production of virus-like particle vaccine protein at gram-per-litre levels, J Biotechnol 150(2) (2010) 224-31.

[27] J.T. Schiller, D.R. Lowy, Vaccines to Prevent Infections by Oncoviruses, Annual Review of Microbiology 64(1) (2010) 23-41.

[28] K. Kwak, A. Yemelyanova, R.B.S. Roden, Prevention of cancer by prophylactic human papillomavirus vaccines, Curr. Opin. Immunol. 23(2) (2011) 244-251.

[29] S. Wynne, R. Crowther, A. Leslie, The crystal structure of the human hepatitis B virus capsid, Molecular cell 3(6) (1999) 771-780.

[30] S.L. Stephen, L. Beales, H. Peyret, A. Roe, N.J. Stonehouse, D.J. Rowlands, Recombinant Expression of Tandem-HBc Virus-Like Particles (VLPs), Methods Mol Biol 1776 (2018) 97-123.

[31] I. Sominskaya, D. Skrastina, A. Dislers, D. Vasiljev, M. Mihailova, V. Ose, D. Dreilina, P. Pumpens, Construction and immunological evaluation of multivalent hepatitis B virus (HBV) core virus-like particles carrying HBV and HCV epitopes, Clin. Vaccine Immunol. 17(6) (2010) 1027-33.

[32] A. Kazaks, I.N. Lu, S. Farinelle, A. Ramirez, V. Crescente, B. Blaha, O. Ogonah, T. Mukhopadhyay, M.P. de Obanos, A. Krimer, I. Akopjana, J. Bogans, V. Ose, A. Kirsteina, T. Kazaka, N.J. Stonehouse, D.J. Rowlands, C.P. Muller, K. Tars, W.M. Rosenberg, Production and purification of chimeric HBc virus-like particles carrying influenza virus LAH domain as vaccine candidates, BMC Biotechnol. 17(1) (2017) 79.

[33] Y. Lei, J. Shao, F. Zhao, Y. Li, C. Lei, F. Ma, H. Chang, Y. Zhang, Artificially designed hepatitis B virus core particles composed of multiple epitopes of type A and O foot-and-mouth disease virus as a bivalent vaccine candidate, J. Med. Virol. 91(12) (2019) 2142-2152.

[34] C.A. Dinarello, J.W. van der Meer, Treating inflammation by blocking interleukin-1 in humans, Semin. Immunol. 25(6) (2013) 469-84.

[35] N. Petrovsky, Comparative Safety of Vaccine Adjuvants: A Summary of Current Evidence and Future Needs, Drug Saf. 38(11) (2015) 1059-1074.

[36] N. Goto, H. Kato, J.-i. Maeyama, K. Eto, S. Yoshihara, Studies on the toxicities of aluminium hydroxide and calcium phosphate as immunological adjuvants for vaccines, Vaccine 11(9) (1993) 914-918.

[37] A. Jegerlehner, M. Wiesel, K. Dietmeier, F. Zabel, D. Gatto, P. Saudan, M.F. Bachmann, Carrier induced epitopic suppression of antibody responses induced by virus-like particles is a dynamic phenomenon caused by carrier-specific antibodies, Vaccine 28(33) (2010) 5503-5512.

[38] J.G. Ogembo, M.R. Muraswki, L.W. McGinnes, A. Parcharidou, R. Sutiwisesak, T. Tison, J. Avendano, D. Agnani, R.W. Finberg, T.G. Morrison, J.D. Fingeroth, A chimeric EBV gp350/220-based VLP replicates the virion B-cell attachment mechanism and elicits long-lasting neutralizing antibodies in mice, J. Transl. Med. 13(1) (2015) 50.

[39] X. Zhang, B. Zhao, M. Ding, S. Song, Y. Kang, Y. Yu, M. Xu, T. Xiang, L. Gao, Q. Feng, Q. Zhao, M.-S. Zeng, C. Krummenacher, Y.-X. Zeng, A novel vaccine candidate based on chimeric virus-like particle displaying multiple conserved epitope peptides induced neutralizing antibodies against EBV infection, Theranostics 10(13) (2020) 5704-5718.

[40] D. Dhanasooraj, R.A. Kumar, S. Mundayoor, Vaccine delivery system for tuberculosis based on nano-sized hepatitis B virus core protein particles, Int J Nanomedicine 8 (2013) 835-43.

[41] R.K. Gupta, Aluminum compounds as vaccine adjuvants, Adv. Drug Del. Rev. 32(3) (1998) 155-172.

[42] A. Bajnok, M. Ivanova, J. Rigó, G. Toldi, The Distribution of Activation Markers and Selectins on Peripheral T Lymphocytes in Preeclampsia, Mediators Inflamm. 2017 (2017) 8045161.

[43] M. Hosono, O.J. de Boer, A.C. van der Wal, C.M. van der Loos, P. Teeling, J.J. Piek, M. Ueda, A.E. Becker, Increased expression of T cell activation markers (CD25, CD26,

CD40L and CD69) in atherectomy specimens of patients with unstable angina and acute myocardial infarction, Atherosclerosis 168(1) (2003) 73-80.

[44] U. Krzych, S. Zarling, A. Pichugin, Memory T cells maintain protracted protection against malaria, Immunology letters 161(2) (2014) 189-195.

[45] F. Sallusto, J. Geginat, A. Lanzavecchia, Central memory and effector memory T cell subsets: function, generation, and maintenance, Annu. Rev. Immunol. 22 (2004) 745-63.

[46] J. Geginat, F. Sallusto, A. Lanzavecchia, Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells, J Exp Med 194(12) (2001) 1711-9.

Chapter 6 Immunogenicity evaluation of chimeric hepatitis B core (HBc) virus-like particle (VLP) displaying Hepatitis C virus core (HCV core) epitope

Abstract

Hepatitis C virus (HCV) is regarded as the cause for the chronic liver diseases leading to a high risk of liver cancer, however, there is no commercial HCV vaccines in the market yet. Production of effective and long-term protective HCV vaccine is necessary. HCV core is the most highly conserved region of the translated HCV genome, making it a promising epitope candidate in the HCV vaccine development. In this project, long and α-helix structured HCV core epitope (aa: 10-53) was inserted at the N-terminus of HBc VLP to form chimeric HCV core-HBc VLP. Designed HCV core-HBc was expressed and purified in the *Escherichia coli* (*E. coli*) expression system under optimized conditions as described in **Chapter 3**. For *in vivo* immunogenicity evaluation, adjuvant free HCV core-HBc VLP is proven to elicit strong epitope specific immune response by animal test. A tendency of Th1 cell derived cellular immune response was detected in the mice immunized with adjuvant free chimeric HCV core-HBc VLP, while another chimeric HBc VLP, EBNA1-HBc, elicits predominant humoral immune response as described in **Chapter 5**. It was found that aluminium adjuvant could affect

the immune response type for HCV core-HBc VLP by improving the epitope specific humoral immune response and suppress the cellular immune response. Finally, similar to EBNA1-HBc VLP, specific memory T cells were detected in the mice after being immunized with HCV core-HBc VLP. This proves that HCV core-HBc VLP obtain the ability to induce long-term immunogenicity *in vivo*. In conclusion, HBc VLP platform is proven to be able to present long and structural antigen and HCV core-HBc VLP is potentially a promising candidate for the treatment of HCV infection.

Key words: Hepatitis C virus; Virus-like particles; Hepatitis B virus core antigen (HBc); Adjuvant free; Long-term immunogenicity

6.1 Introduction

Liver cancer is a malignant tumour that grows in the liver in three different types including hepatocellular carcinoma (HCC), cholangiocarcinoma, and angiosarcoma[1]. It has been reported that chronic infection with hepatitis C virus and hepatitis B virus could lead to the development of HCC[2, 3]. Recently, vaccines based on HBV was developed and among them, Engerix-B (ENG) and Recombivax HB (REC) are the two FDA-approved vaccines that prevent the infection of HBV. However, there is no reported HCV vaccines for liver cancer treatment yet.

Based on the process for the successful development of hepatitis B virus and human papillomavirus vaccines[4, 5], recombinant chimeric virus-like particle (VLP) based vaccines for HCV related diseases and cancers could be a promising approach to develop effective vaccines against HCV. Virus-like particles (VLPs) have been proven to be one of the candidates for the development of cancer vaccine delivery platform in last decade[6]. Various VLPs from different viruses have been studied and evaluated to be adopted for presenting foreign epitopes[7-9].

Hepatitis B virus core antigen (HBc) is one of the most popular VLP carriers that have been developed and has been regarded as the most flexible model for foreign peptides presentation in the last decade[10]. HBc VLP has been developed as the promising vaccine platform for presenting foreign epitopes due to its high-level synthesis and efficient self-assembly in all expression system[11]. In the application of HBc VLP platform for presenting foreign epitopes, short epitopes (less than 20 amino acids) are

chosen as they are considered to have less impact on the structure and stability of chimeric HBc VLPs[12]. Several chimeric HBc VLPs have been developed by using HBc VLP to present short epitopes such as SP55 (PDSRESLAWQTATNP) and SP70 (YPTFGEHKQEKDLEY) epitope of EV71[13] and HCC epitopes MAGE-1(278-286aa), MAGE-3(271-279aa), AFP1 (158-166aa) or AFP2 (542-550aa)[14]. However, challenges for soluble expression of these designed chimeric HBc VLPs remained. In **Chapter 5**, Hepatitis B core virus like particle (HBc VLP) is able to present short and non-structural antigen with stable assembled VLP structure and elicit strong and long-term immune response against the specific antigen. Recently, several strategies have been developed to improve the ability of HBc VLP to present large foreign antigen sequence and these strategies includes the SplitCore[15], the use of a non-covalent "binding-tag" peptide[16], and the tandem core technology[17]. However, all these strategies focus on the insertion of foreign epitopes in the major immunodominant region (MIR) of HBc and there is no report about the study for insertion of large foreign antigens at N-terminal of HBc VLP yet.

In this chapter, HCV core epitope (aa 10-53) was designed to fused at the N-terminus of HBc VLP for the evaluation. HCV core was reported to be involved in the activation of several cellular signalling pathways of HCV. Moreover, HCV core plays a crucial role in the regulation of the gene expression of HCV leading to HCC[18]. This makes HCV core antigen to be a promising target for the treatment of HCV related diseases and cancers. The HCV core epitope selected in our project is composed with 44 amino

acids including 12 positively charged amino acids and it also contains the structure of α-helix. The designed recombinant chimeric HCV core-HBc VLP was expressed using optimized *E. coli* expression system and purified with optimized ammonium sulphate precipitation process as described in **Chapter 3**. *In vivo* immunogenicity of chimeric HCV core-HBc VLP with and without adjuvant was evaluated to investigate the performance of chimeric HBc VLP when presenting long and structural antigen on the surface. In addition, long-term immunogenicity of chimeric HCV core-HBc VLP was examined by the measurement of memory T cells.

6.2 Materials and Methods

6.2.1 Expression and purification of HCV core-HBc VLP

Chimeric HCV core-HBc VLPs were expressed and purified with the optimized condition as described in **Chapter 3**.

6.2.2 Immunization scheme and immunogenicity evaluation

Similar to the scheme in **Chapter 5**, female BALB/c mice aged at 6–8 weeks (body weight about 18–20 g) were purchased from SPF Biotechnology Co., Ltd, (Beijing, China) and maintained with pathogen-free water and food. The animals were randomly divided into 6 groups for 8 animals per group. The six groups were 1) control groups: PBS (negative control), OVA (positive control), HCV core peptide group, wt HBc VLP and 2) sample groups: HCV core-HBc VLP without adjuvant, and HCV core-HBc with aluminium hydroxide adjuvant (SERVA Electrophoresis GmbH, Germany) groups. For

sample group, the mice were immunized intraperitoneally with 100 μg of samples in 200 μl of sterile PBS on days 0, 14, and 28. For HCV core peptide group, 19.4 μg of HCV core peptide in 200 μl of sterile PBS was injected on days 0, 14, and 28. For HBc VLP group, 80.6 μg of HBc VLP in 200 μl of sterile PBS was injected on days 0, 14, and 28. For evaluation of the humoral immune response, immune serum was collected 10 days after first and second boost. For T-cell proliferation measurement and lymphocytes experiments, mice were sacrificed, and spleens were obtained at day 38 after the first immunization. Sera were isolated and stored at -70 ℃ until use. The design and progress of immunogenicity evaluation of HCV core-HBc VLP including ELISA for antibody titer, T-cell proliferation assay, Cytokine test, Lymphocyte activation evaluation, and Memory T cells activation assay were the same as described in **Chapter 5**.

6.2.3 Statistical analysis

Statistical results were analysed using GraphPad-Prism 6 software (Graph- Pad Software, USA). The values of $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were considered statistically significant between the experimental groups, respectively.

6.2.4 Ethical statement

This study was approved by the Animal Ethics Committee of Shanxi University of Chinese Medicine (Shanxi, China Approval number: 2019LL137).

6.3 Results

6.3.1 Humoral response in mice

Serum of mice vaccinated with HCV core-HBc VLP was collected on day 24 and 38 after initial immunization for immunogenicity evaluation. Reference groups were immunized with wt HBc VLP and HCV core peptide. As shown in **Figure 6.1**, the 2nd boost of vaccination resulted in a significant improved HCV core specific antibody titer of HCV core-HBc VLP groups with and without adjuvant compared with HCV core peptide group. The epitope specific antibody titer achieved by adjuvant free HCV core-HBc was 9.1×10^4 after 2nd boost while the antibody titer achieved by the group with aluminium adjuvant achieved by 2.9×10^5. This suggested that aluminium adjuvant potentially have large improvement for the humoral immune response of HCV core-HBc VLP. However, HBc VLP specific antibody titers were detected in both HCV core-HBc VLP groups with and without adjuvant. This indicated that when presenting foreign epitope with HBc VLPs, carrier specific immune response was unavoidable.

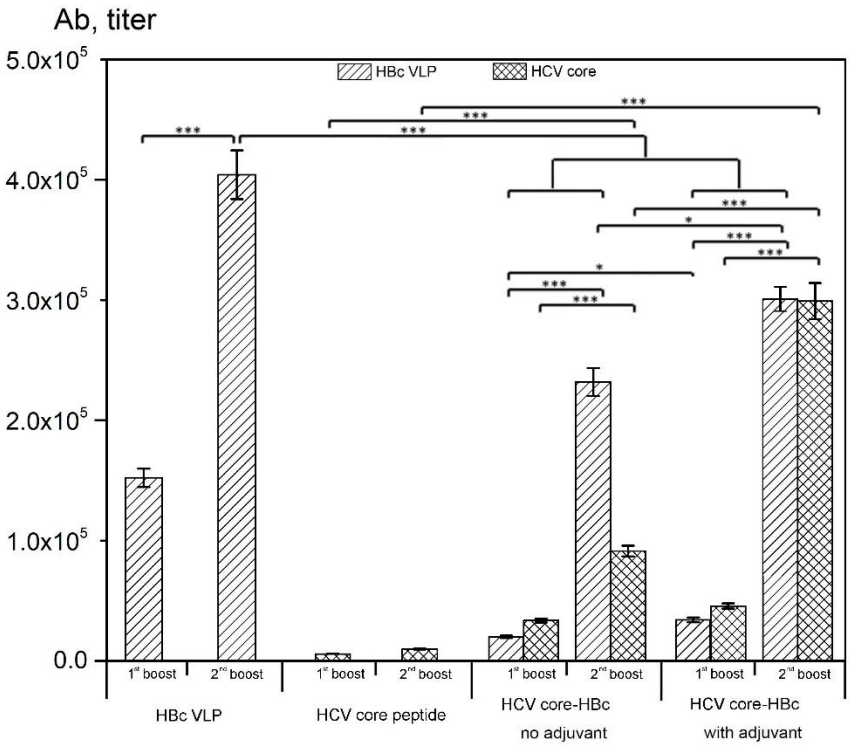

Figure 6.1 Induction of antibodies in mice by chimeric HBc-derived VLPs. Shown are antibody titers in pools of sera from eight animals at day 24 (1st boost) and at day 38 (2nd boost) after the first immunization. Titers in specimens were determined on plate coated with HBc VLP and HCV core peptide. Antibody titers of mice treated with HCV core peptide and HBc VLP were used as references. Data are expressed as mean ± SD (n=8) (*p<0.05; **p<0.01; ***p<0.001).

6.3.2 Cellular immune response and cytokine assay

There are specific antigen receptors on the surface of B cells and T cells. With this property, lymphocytes proliferated by the stimulation of specific antigens, and the proliferation of lymphocytes was used to evaluate the cellular immune response of the vaccines[19]. Lymphocytes of mice immunized with chimeric HCV core-HBc VLP with and without adjuvant demonstrated detectable proliferation after being stimulated with HCV core peptide and HBc VLP *in vitro* at day 38 after the first immunization (**Figure 6.2**). Both chimeric HCV core-HBc VLPs with and without adjuvant groups showed a significant increase of HCV core peptide specific proliferation *in vitro*,

compared with the group immunized with HCV core peptide, where adjuvant free HCV core-HBc group achieved around 6 times of the T-cell proliferation and the group with adjuvant achieved around three times of T-cell proliferation compared with HCV core peptide group.

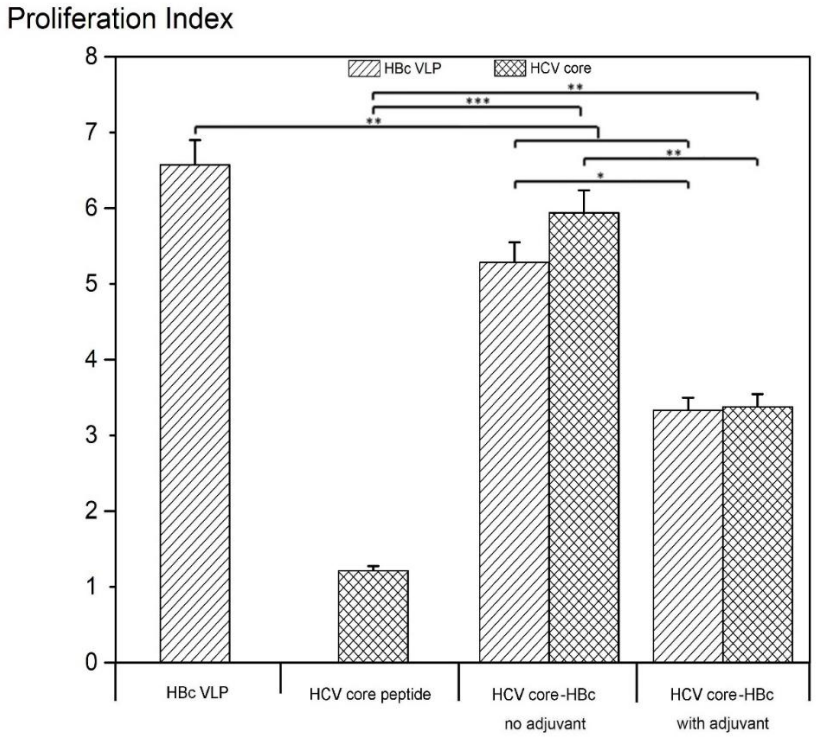

Figure 6.2 Lymphocyte proliferation after immunization of mice by HCV core-HBc VLPs, wt HBc VLP and HCV core peptide. PI were measured in response to stimulation of T cells with HBc VLP and HCV core peptide. The error bars indicate standard deviations. Proliferation results for T-cells of mice treated with HCV core peptide and HBc VLP were used as references. Data are expressed as mean ± SD (n=8) (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

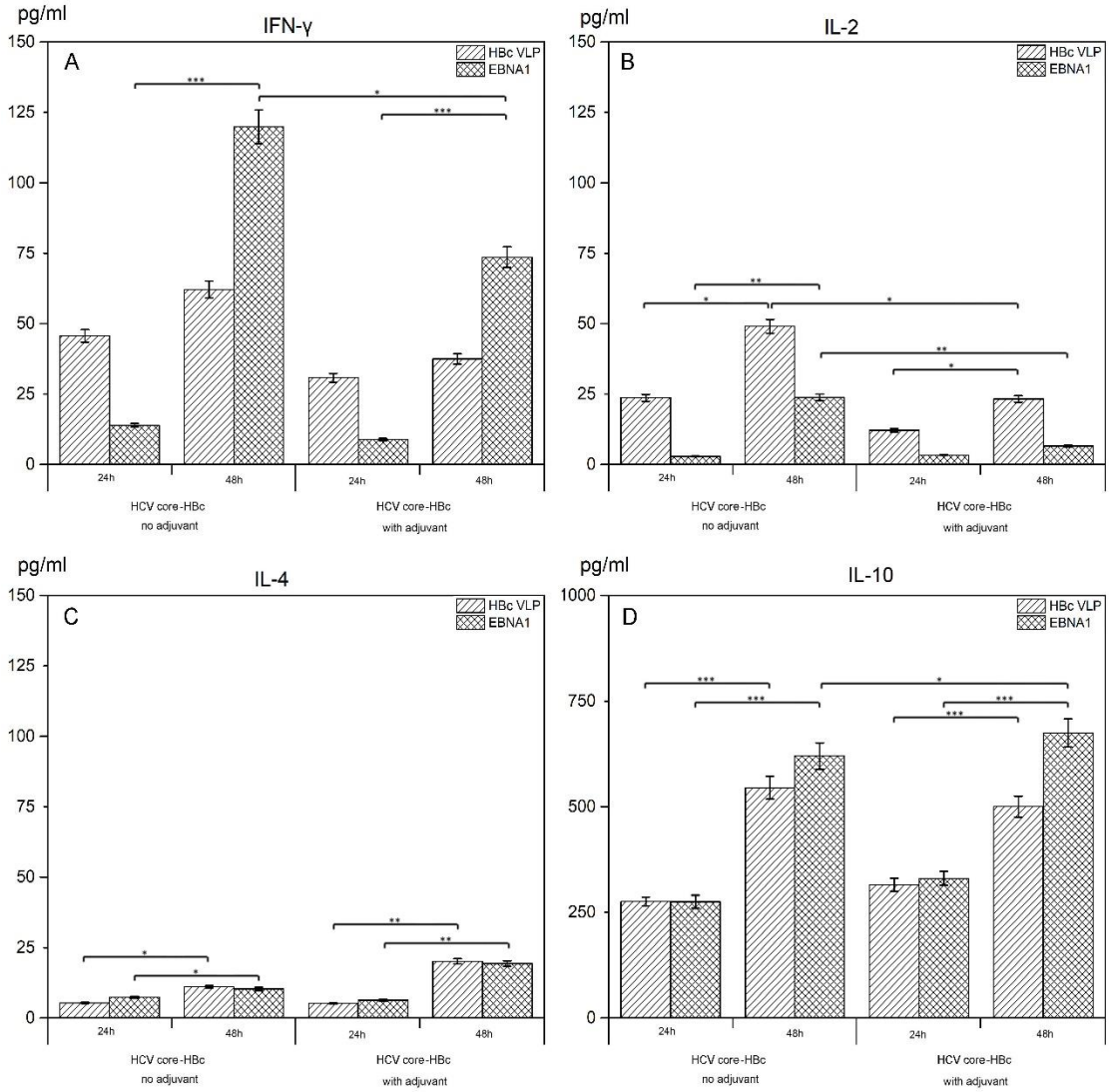

Figure 6.3 Cytokines produced by T cells after immunization of mice with HCV core-HBc VLPs. Cell supernatants were removed and analysed at 24 h and 48 h for IFN-γ(a), IL-2 (b), IL 4 (c) and IL-10 (d) production after stimulation by HBc VLP and HCV core peptide. Data are expressed as mean ± SD (n=8) (*p<0.05; **p<0.01; ***p<0.001).

Figure 6.3 illustrates the detected concentration of the Th1 cell-derived cytokines IFN-γ, IL-2, and IL-10 after stimulation with HCV core peptide and HBc VLP for 24 h and 48 h. The observed results matched with previous lymphocytes proliferation examination. All cytokine expression levels were increased after 48 h culture compared with the one with 24 h culture due to the proliferation and activation of lymphocytes after being stimulated by HCV core peptide. After 48 h, amongTh1 cell derived

cytokines, the detected amount of IL-2 was relatively low (23.7 pg/ml for adjuvant free group and 11.2 pg/ml for the group with adjuvant) compared with that of IFN-γ (119.8 pg/ml for adjuvant free group and 73.6 pg/ml for the group with adjuvant) and IL-10 (620 pg/ml for adjuvant free group and 674.5 pg/ml for the group with adjuvant). Th2 cell-derived cytokine IL-4 were also measured, and the amount of IL-4 was detectable, but much lower than all Th1 cell-derived cytokines (10.3 pg/ml for adjuvant free group and 19.3 pg/ml for the group with adjuvant). After the addition of adjuvant, the detected Th1 cell-derived cytokines IFN-γ and IL-2 were lower than those of adjuvant free group, while the detected amount of Th2 cell-derived cytokine, IL-4 was slightly higher. This suggests that the aluminium adjuvant had the potential to regulate the immune response of chimeric HCV core-HBc VLP to a Th2 cell derived immune response and potentially improve the humoral immune response of HCV core-HBc VLP.

6.3.3 IgG isotype of HCV core-HBc VLP

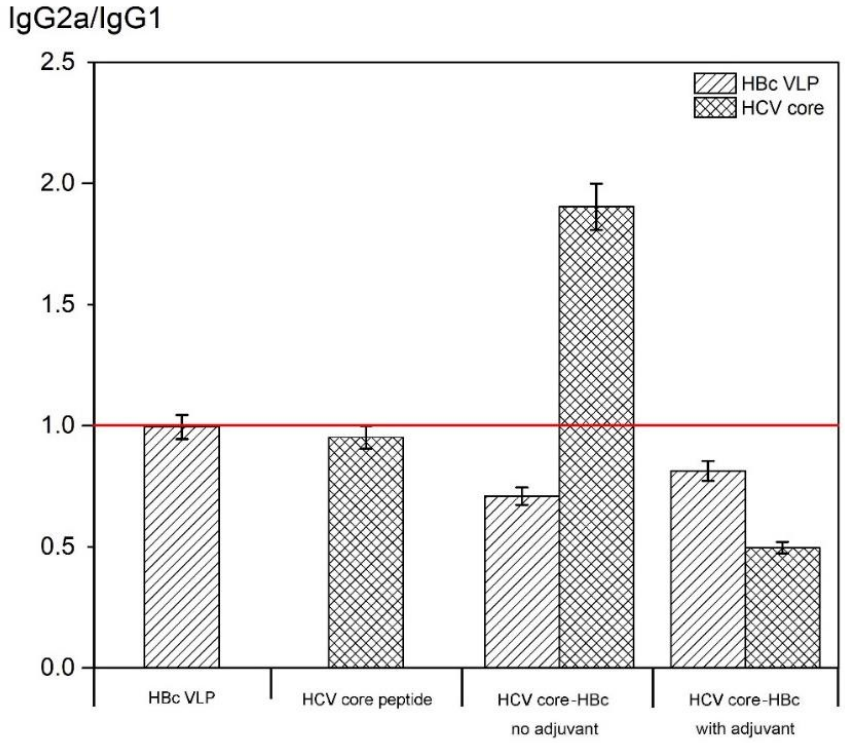

Figure 6.4 IgG isotype distribution in serum collected 10 days after 2nd boost vaccination of mice by HCV core-HBc VLP with and without adjuvant. The red line depicts the equivalent distribution of IgG2a and IgG1 isotypes.

The IgG isotype in the sera of mice immunized with HCV core-HBc VLP with and without adjuvant was examined using ELISA. HCV core peptide and wt HBc VLP groups were used as references. As shown in **Figure 6.4**, both reference groups showed a IgG2a/IgG1 ratio at around 1. Adjuvant free HCV core-HBc VLP group showed a predominated HCV core specific IgG2a in the immunized mice. This indicates that adjuvant free HCV core-HBc VLP elicited predominated Th1 cell derived immune response *in vivo*. After addition of aluminium adjuvant, it was found that the predominated IgG type changed to IgG1 which represents a predominated Th2 cell derived immune response. This finding matches with our previous finding in the cytokine assay of HCV core-HBc VLP. However, as indicated in **section 6.3.1,** carrier

induced immune response still existed and the carrier specific immune response remained as predominated Th2 cell derived immune response.

6.3.4 Lymphocyte activation

To further review the immune response of chimeric HCV core-HBc VLP, the percentage of activated lymphocytes was examined in $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells using the flowcytometry (**Figure 6.6**). CD25 marker was applied as the active marker for the detection[20]. As shown in **Figure 6.5**, chimeric HCV core-HBc VLP with and without adjuvant groups showed a significant increase in the percentage of activated $CD19^+$ B cells, $CD4^+$ T cells and $CD8^+$ T cells after being stimulated with HCV core peptide. The percentage of $CD19^+$ B cells, $CD4^+$ T cells and $CD8^+$ T cells induced by adjuvant free HCV core-HBc increased from 0.7 %, 0.5 % and 1.2 % to 2.5 %, 6.1 % and 5.5 %, respectively. This indicates that chimeric HCV core-HBc VLP potentially induced strong cellular immune response. After adding the adjuvant, the percentages of activated T cells were found to be lower than the adjuvant free group while the percentages of activated B cells was higher.

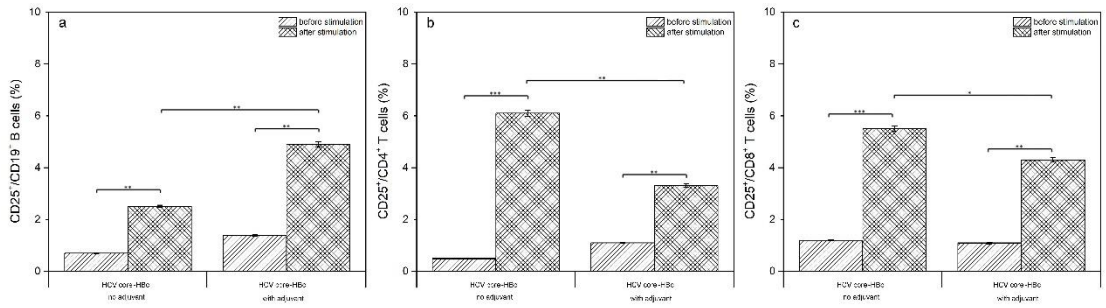

Figure 6.5 Activation of $CD19^+$ B cells (a), $CD4^+$ T cells (b) and $CD8^+$ T cells (d) of lymphocytes after immunization of mice with HCV core-HBc VLPs stimulated by HCV core peptide. Data are expressed as mean ± SD (n=3) (*p<0.05; **p<0.01; ***p<0.001).

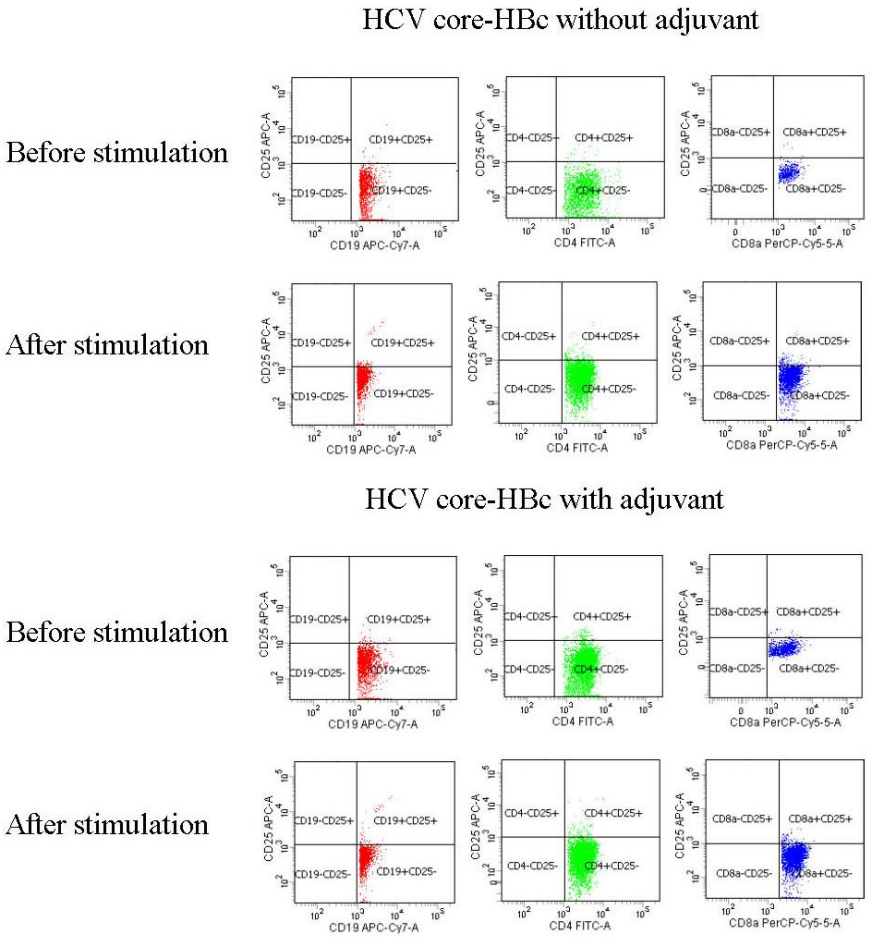

Figure 6.6 Flow cytometry of CD25 marker in $CD19^+$ B cells, $CD4^+$ T cells and $CD8^+$ T cells of lymphocytes after immunization of mice with HCV core-HBc VLPs stimulated by HCV core peptide.

6.3.5 Memory T cells

Long-term protection is the ultimate goal for successful vaccines. To evaluate the long-term protection property of HCV core-HBc VLP, the percentage of memory $CD4^+$ and $CD8^+$ T cells were evaluated. CD44 and CD62L are two major markers on the surface of memory T cells and have been applied in our previous work. On the surface of central memory T cells (T_{CM} cells), CD44 and CD62L makers are highly expressed, while on the surface of effector memory T cells (T_{EM} cells), CD44 maker is highly expressed and

CD62L is expressed in low level. The surface markers of $CD4^+$ and $CD8^+$ T cells of the mice vaccinated with HCV core-HBc VLP were evaluated with flowcytometry (**Figure 6.8**). As shown in **Figure 6.7**, the percentage of $CD4^+$ and $CD8^+$ T_{CM} cells induced by adjuvant free HCV core-HBc VLP decreased from 3 % and 6.8 % to 1 % and 2.6 %, respectively, and the percentage of $CD4^+$ and $CD8^+$ T_{EM} cells increased from 12 % and 17.6 % to 34.5 % and 27.4 %, respectively. It is also observed that $CD8^+$ T_{CM} cells produced in adjuvant free HCV core-HBc VLP group was higher than that produced by the group with aluminium adjuvant. This demonstrates the successful production of the central memory T cells targeting against HCV core peptide after immunized with produced HCV core-HBc VLP.

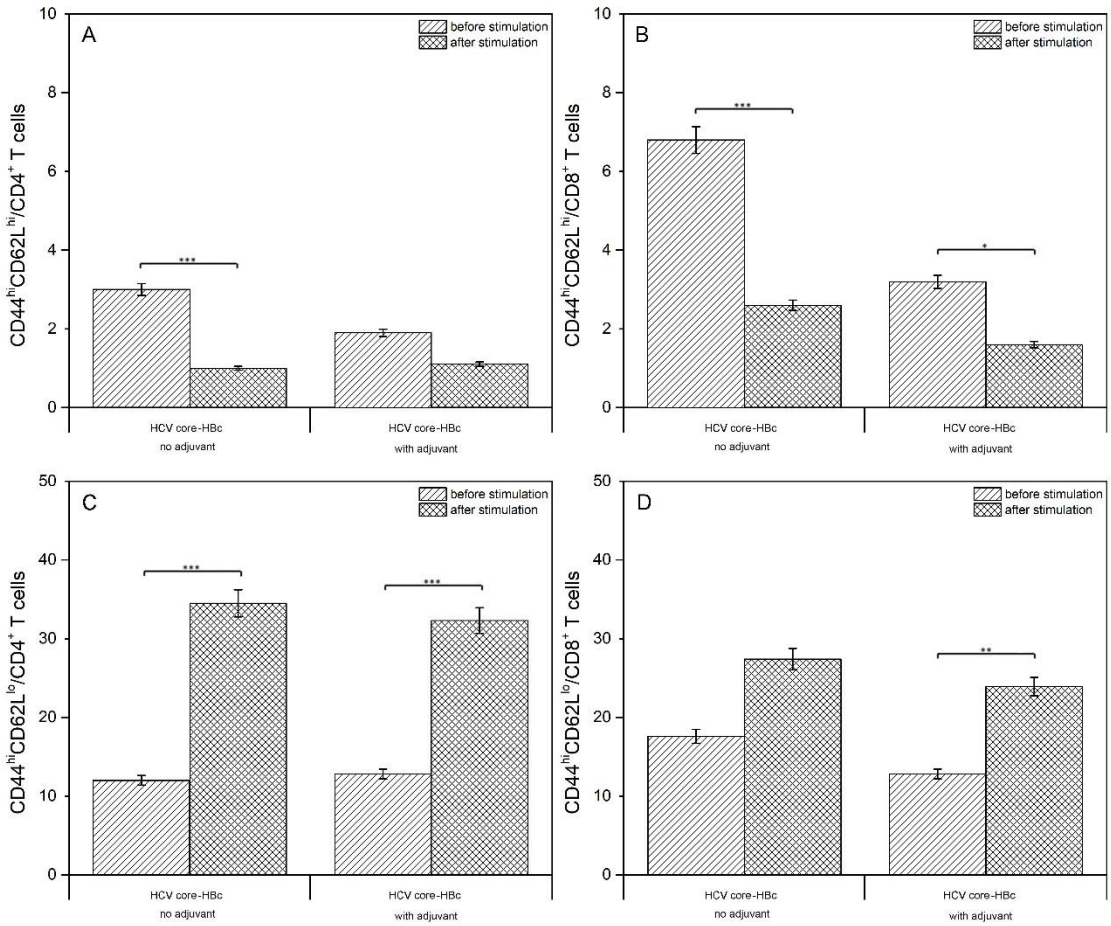

Figure 6.7 Measurement of central ($CD44^{hi}CD62L^{hi}$)/effector ($CD44^{hi}CD62L^{low}$) memory $CD4^+$ and $CD8^+$ T cells. Splenocytes were harvested on day 38 after the first immunization and stimulation *in vitro* by HCV core peptide for 60 h. The frequency of CD44hiCD62Lhi/$CD4^+$ T cells, $CD44^{hi}CD62L^{low}/CD4^+$ T cells, $CD44^{hi}CD62L^{hi}/CD8^+$ T cells, and $CD44^{hi}CD62L^{low}/CD8^+$ T cells were measured by flow cytometry. Data are expressed as mean ± SD (n=3) (*p<0.05; **p<0.01; ***p<0.001).

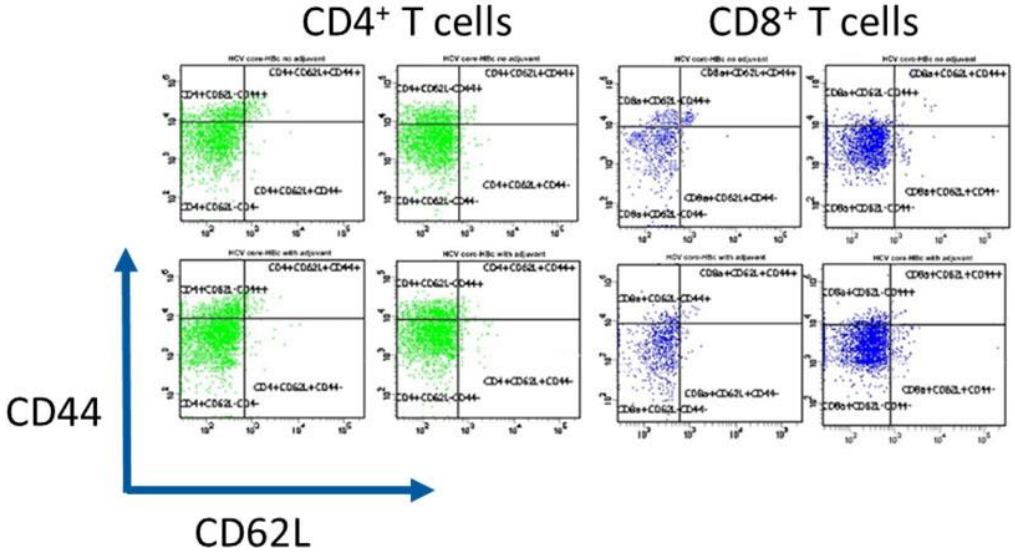

Figure 6.8 Flow cytometry of central/effector memory $CD4^+$ and $CD8^+$ T cells.

6.4 Discussion

Hepatitis C virus (HCV) is regarded as the cause for chronic infection leading to the liver cancers. To treat HCV infection, an effective preventive vaccine with long-term protection is required. However, up to date, there is no commercial HCV vaccine available yet. In our project, the aim is to construct an effective and long-lasting preventive HCV vaccine by presenting the long and structural HCV core epitope using HBc VLP platform to form chimeric HCV core-HBc VLP. HCV core-HBc VLP proteins were expressed and purified under optimized conditions as described in **Chapter 3**. The purified HCV core-HBc VLP proteins were dialysed against 20 mM PB, pH 7.4 for evaluation of *in vivo* immunogenicity.

In the immunogenicity evaluation by animal test, both HCV core-HBc VLP groups with and without adjuvant induced significantly improved HCV core specific antibody titer compared with HCV core peptide group after second boost. However, the HCV core specific antibody titer achieved by HCV core-HBc VLP group with aluminium adjuvant was significantly higher than that of adjuvant free HCV core-HBc VLP group. However, the achieved epitope specific antibody titer level of 9.1×10^4 by adjuvant free group is still higher than other reported HCV vaccine candidates produced by Sominskaya's group (antibody titer less than 2,000)[21] and is similar to a recent reported HCV VLP containing the HCV core and the E1 and E2 envelope glycoproteins produced by Christiansen's group (antibody titer level at around 10^4)[22]. In the cellular immune response evaluation, adjuvant free HCV core-HBc VLP achieved a six times

higher cellular immune response compared with HCV core peptide group and twice higher cellular immune response compared with HCV core-HBc VLP with adjuvant group. The lower cellular immune response achieved by HCV core-HBc VLP with adjuvant group indicates that aluminium adjuvant may cause the suppression of cellular immune response induced by HCV core-HBc. These findings in humoral and cellular immune response induced by HCV core-HBc VLP groups indicate that the immune response type of HCV core-HBc VLP can be largely influenced by the addition of aluminium adjuvant while it has minor influence on the immune response type of our previously produced EBNA1-HBc VLP in **Chapter 5**. For HCV core-HBc VLP, aluminium adjuvant can significantly suppress the cellular immune response while also significantly improve the humoral immune response of chimeric HCV core-HBc VLP at the same time. Cytokine assay results also indicate that a higher level of cellular immune response related cytokines was produced in adjuvant free HCV core-HBc VLP group compared with the group with aluminium adjuvant. These findings match with IgG isotype assay where predominated Th2 cell derived HCV core specific humoral immune response was found for HCV core-HBc VLP group with aluminium adjuvant and adjuvant free HCV core-HBc VLP group induced predominated Th1 cell derived HCV core specific cellular immune response[23]. In addition, similar to our previous finding in **Chapter 5**, carrier induced epitopic suppression (CIES) effect[24] is also found in the immune response induced by both HCV core-HBc VLP groups with and without adjuvant. The total antibody titers achieved by HCV core-HBc VLP groups

were lower compared with that achieved by wt HBc VLP group. Report has suggested that higher coupling densities, repeated injections or higher doses of vaccination could be applied to solve this issue[24]. Chimeric HCV core-HBc VLP showed a weaker self-adjuvant effect compared with other reported vaccines[9, 25, 26] and also our produced EBNA-HBc VLP. This could be caused by the impact on the structure and the property of VLPs after insertion of HCV core epitope.

The activation of T cells and B cells of HCV core-HBc VLP with and without adjuvant groups after stimulation with HCV core peptide was examined to further evaluate the immune response induced by HCV core-HBc VLPs. Similar to our previous work, CD25 maker was used the activation marker for T cells and B cells[20, 27]. Both HCV core-HBc VLP groups with and without aluminium adjuvant observed a significant increase of the percentage of activated $CD19^+$ B cells, $CD4^+$ cells and $CD8^+$ T cells after stimulation with HCV core peptide. High percentage of activated $CD4^{+\,T}$ cell and $CD8^+$ T cells were achieved by 6.1 % and 5.5 %, respectively. This matches with our previous findings that adjuvant free HCV core-HBc VLPs elicits predominated cellular immune response and it also proves that chimeric HCV core-HBc VLP is promising to induce strong epitope specific immune response.

Finally, HCV core specific long-term immunogenicity was detected by measuring the central memory T cells. Similar to our previous work in **Chapter 5**, two types of memory T cells, central memory T cells (T_{CM} cells) longer lifetime but the slower respond and effective memory T cells (T_{EM} cells) with shorter lifetime but rapid respond,

were measured using flowcytometry[28]. HCV core peptide was used as the stimulator to examine the specific response of memory T cells against HCV core peptide. Results indicate that mice immunized with both HCV core-HBc VLP groups with and without aluminium adjuvant group are able to produce $CD4^+$ T_{CM} cells, $CD4^+$ T_{EM} cells, $CD8^+$ T_{CM} cells and $CD8^+$ T_{EM} cells. Higher percentage of $CD8^+$ T_{CM} cells was observed in adjuvant free HCV core-HBc VLP group as it triggers predominated Th1 cell derived cellular immune response. After being stimulated with HCV core peptide, the percentage of $CD4^+$ T_{CM} cells and $CD8^+$ T_{CM} cells of both HCV core-HBc VLP groups with and without aluminium adjuvant decreased. In addition, higher percentage of $CD8^+$ T_{CM} cells (6.8 %) produced with adjuvant free HCV core-HBc VLP vaccinated mice was detected compared with that (3.5 %) produced by adjuvant free EBNA1-HBc VLP vaccinated mice as described in **Chapter 5**. This could be explained by the stronger cellular immune response of HCV core-HBc VLP compared with EBNA1-HBc VLP. Memory T cell assay result demonstrates that the produced T_{CM} cells have specific response against HCV core peptide and indicates that chimeric HCV core-HBc VLP obtain the long-term protection ability against HCV core peptide.

6.5 Conclusion

In this chapter, 44-mer HCV core epitope with an α-helix was successfully fused into the N-terminus of HBc VLP to form chimeric HCV core-HBc VLP. The expression and purification process were followed by our previous work in **Chapter 3**. High purity with 96 % and high production yield with 40.4 mg/g of wet cell weight were achieved.

In vivo immunogenicity evaluation demonstrates that adjuvant free HCV core-HBc VLP is able to elicit strong HCV core specific immune response and it has the tendency to induce predominated cellular immune response. However, after addition with aluminium adjuvant, the tendency of immune response changes to predominated humoral immune response. The influence of aluminium adjuvant on the immune response performance is critical compared with other HBc derived VLP vaccines and our EBNA1-HBc VLP when presenting short and non-structural antigen using HBc VLP platform. This finding indicates that when displaying long and complicated antigens on HBc VLP platform, the immunogenicity of induced by chimeric HBc VLP is easy to be influenced by other factors such as the property of the adjuvant and the nature of the presented antigen. In addition to elicit strong immune response, adjuvant free HCV core-HBc VLP is proven to induce high percentage of memory T cells with 3 % for $CD4^+$ T_{CM} cells and 6.8 % $CD8^+$ T_{CM} cells. These findings indicate that HBc VLP platform can be applied to present long and structural foreign antigen at N-terminus and the produced adjuvant free HCV core-HBc VLP is promising to be applied as the vaccine candidate for the treatment of HCV infection. Further HCV neutralization assay is needed to confirm that HCV core-HBc VLP is a suitable vaccine candidate for the treatment of HCV infection.

References

[1] Y. Kim, A. Ejaz, A. Tayal, G. Spolverato, J.F.P. Bridges, R.A. Anders, T.M. Pawlik, Temporal trends in population-based death rates associated with chronic liver disease and liver cancer in the United States over the last 30 years, Cancer 120(19) (2014) 3058-3065.

[2] G. Fattovich, G. Giustina, F. Degos, F. Tremolada, G. Diodati, P. Almasio, F. Nevens, A. Solinas, D. Mura, J.T. Brouwer, H. Thomas, C. Njapoum, C. Casarin, P. Bonetti, P. Fuschi, J. Basho, A. Tocco, A. Bhalla, R. Galassini, F. Noventa, S.W. Schalm, G. Realdi, Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients, Gastroenterology 112(2) (1997) 463-72.

[3] R.P. Beasley, Hepatitis B virus. The major etiology of hepatocellular carcinoma, Cancer 61(10) (1988) 1942-56.

[4] J.T. Schiller, D.R. Lowy, Vaccines to Prevent Infections by Oncoviruses, Annual Review of Microbiology 64(1) (2010) 23-41.

[5] K. Kwak, A. Yemelyanova, R.B.S. Roden, Prevention of cancer by prophylactic human papillomavirus vaccines, Curr. Opin. Immunol. 23(2) (2011) 244-251.

[6] N.K.C. Linda H.L. Lua, Frank Sainsbury, Yap P. Chuan,Nani Wibowo, Anton P.J. Middelberg, Bioengineering Virus-Like Particles as Vaccines, Biotechnology and Bioengineering 111 (2014) 425-440.

[7] M.O. Mohsen, D.E. Speiser, A. Knuth, M.F. Bachmann, Virus-like particles for vaccination against cancer, Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology n/a(n/a) e1579.

[8] D. Yan, Y.-Q. Wei, H.-C. Guo, S. Sun, The application of virus-like particles as vaccines and biological vehicles, Applied microbiology and biotechnology 99 (2015).

[9] H.K. Ong, W.S. Tan, K.L. Ho, Virus like particles as a platform for cancer vaccine development, PeerJ 5 (2017) 4053-4053.

[10] K. Roose, S. De Baets, B. Schepens, X. Saelens, Hepatitis B core-based virus-like particles to present heterologous epitopes, Expert Rev Vaccines 12(2) (2013) 183-98.

[11] S.L. Stephen, L. Beales, H. Peyret, A. Roe, N.J. Stonehouse, D.J. Rowlands, Recombinant Expression of Tandem-HBc Virus-Like Particles (VLPs), Methods Mol Biol 1776 (2018) 97-123.

[12] K. Roose, S.D. Baets, B. Schepens, X. Saelens, Hepatitis B core–based virus–like particles to present heterologous epitopes, Expert review of vaccines 12(2) (2013) 183-198.

[13] X. Ye, Z. Ku, Q. Liu, X. Wang, J. Shi, Y. Zhang, L. Kong, Y. Cong, Z. Huang, Chimeric Virus-Like Particle Vaccines Displaying Conserved Enterovirus 71 Epitopes Elicit Protective Neutralizing Antibodies in Mice through Divergent Mechanisms, J. Virol. 88(1) (2014) 72.

[14] Y. Zhang, S. Song, C. Liu, Y. Wang, X. Xian, Y. He, J. Wang, F. Liu, S. Sun, Generation of chimeric HBc proteins with epitopes in E.coli: Formation of virus-like particles and a potent inducer of antigen-specific cytotoxic immune response and anti-tumor effect in vivo, Cellular Immunology 247(1) (2007) 18-27.

[15] A. Walker, C. Skamel, M. Nassal, SplitCore: An exceptionally versatile viral nanoparticle

for native whole protein display regardless of 3D structure, Sci. Rep. 1(1) (2011) 5.
[16] E.A. Blokhina, V.V. Kuprianov, L.A. Stepanova, L.M. Tsybalova, O.I. Kiselev, N.V. Ravin, K.G. Skryabin, A molecular assembly system for presentation of antigens on the surface of HBc virus-like particles, Virology 435(2) (2013) 293-300.
[17] H. Peyret, A. Gehin, E.C. Thuenemann, D. Blond, A. El Turabi, L. Beales, D. Clarke, R.J.C. Gilbert, E.E. Fry, D.I. Stuart, K. Holmes, N.J. Stonehouse, M. Whelan, W. Rosenberg, G.P. Lomonossoff, D.J. Rowlands, Tandem fusion of hepatitis B core antigen allows assembly of virus-like particles in bacteria and plants with enhanced capacity to accommodate foreign proteins, PLoS One 10(4) (2015) e0120751-e0120751.
[18] S. Jahan, S. Khaliq, B. Ijaz, W. Ahmad, S. Hassan, Role of HCV Core gene of genotype 1a and 3a and host gene Cox-2 in HCV-induced pathogenesis, Virol J. 8(1) (2011) 155.
[19] S. Datta, N. Sarvetnick, Lymphocyte proliferation in immune-mediated diseases, Trends Immunol. 30(9) (2009) 430-438.
[20] M. Hosono, O.J. de Boer, A.C. van der Wal, C.M. van der Loos, P. Teeling, J.J. Piek, M. Ueda, A.E. Becker, Increased expression of T cell activation markers (CD25, CD26, CD40L and CD69) in atherectomy specimens of patients with unstable angina and acute myocardial infarction, Atherosclerosis 168(1) (2003) 73-80.
[21] I. Sominskaya, D. Skrastina, A. Dislers, D. Vasiljev, M. Mihailova, V. Ose, D. Dreilina, P. Pumpens, Construction and immunological evaluation of multivalent hepatitis B virus (HBV) core virus-like particles carrying HBV and HCV epitopes, Clin. Vaccine Immunol. 17(6) (2010) 1027-33.
[22] D. Christiansen, L. Earnest-Silveira, B. Grubor-Bauk, D.K. Wijesundara, I. Boo, P.A. Ramsland, E. Vincan, H.E. Drummer, E.J. Gowans, J. Torresi, Pre-clinical evaluation of a quadrivalent HCV VLP vaccine in pigs following microneedle delivery, Sci. Rep. 9(1) (2019) 9251-9251.
[23] A. Berger, Th1 and Th2 responses: what are they?, BMJ (Clinical research ed.) 321(7258) (2000) 424-424.
[24] A. Jegerlehner, M. Wiesel, K. Dietmeier, F. Zabel, D. Gatto, P. Saudan, M.F. Bachmann, Carrier induced epitopic suppression of antibody responses induced by virus-like particles is a dynamic phenomenon caused by carrier-specific antibodies, Vaccine 28(33) (2010) 5503-5512.
[25] V. Cimica, J.M. Galarza, Adjuvant formulations for virus-like particle (VLP) based vaccines, Clinical immunology (Orlando, Fla.) 183 (2017) 99-108.
[26] G. Guillén, J.C. Aguilar, S. Dueñas, L. Hermida, M.G. Guzmán, E. Penton, E. Iglesias, J. Junco, I. Torrens, Y. Lobaina, V. Muzio, L. Herrera, Virus-Like Particles as vaccine antigens and adjuvants: Application to chronic disease, cancer immunotherapy and infectious disease preventive strategies, Procedia in Vaccinology 2(2) (2010) 128-133.
[27] A. Bajnok, M. Ivanova, J. Rigó, G. Toldi, The Distribution of Activation Markers and Selectins on Peripheral T Lymphocytes in Preeclampsia, Mediators Inflamm. 2017 (2017) 8045161.
[28] F. Sallusto, J. Geginat, A. Lanzavecchia, Central memory and effector memory T cell subsets: function, generation, and maintenance, Annu. Rev. Immunol. 22 (2004) 745-63.

Chapter 7 Conclusions and future directions

7.1 Conclusions

In conclusion, two chimeric HBc VLPs, chimeric EBNA1-HBc VLP and HCV core-HBc VLP have been optimized to be expressed using the *E. coli* expression system and purified with ammonium sulphate precipitation with high production yields. In the property and stability examination of chimeric HBc VLPs presenting different epitopes, chimeric EBNA1-HBc VLP shows a similar stability when being treated with different temperature and freeze/thaw cycles compared with wt HBc VLP. Lower tolerances were detected in treatment of high concentration of SDS and low pH value. However, when presenting long and structured epitope, HCV core, with HBc VLP, chimeric HCV core-HBc shows a much worse stability compared with EBNA1-HBc VLP and wt HBc VLP. With the help of computational protein modelling, it is found that the hydrophobicity of chimeric HBc monomers has great relationship with the stability of chimeric HBc VLPs as the hydrophobic interaction between monomers is vital for the assembly of chimeric HBc VLPs. More epitopes need to be examined to confirm this finding.

In the immunogenicity evaluation, both adjuvant free chimeric EBNA1-HBc VLP and HCV core-HBc VLP elicit strong epitope specific immune response compared with other reported vaccines against EBV and HCV. A predominated humoral immune response is detected in the mice vaccinated with chimeric EBNA1-HBc VLP while mice immunized with chimeric HCV core-HBc VLP shows a predominated cellular immune response. The different immune response performance and tendency is potentially caused by the nature of epitopes presented by HBc VLP. In addition, aluminium adjuvant is able to improve the humoral immune response of both chimeric HBc VLP vaccines due to the propensity of aluminium adjuvant to induce IL-1. However, less impact of aluminium adjuvant on the immune response of chimeric EBNA1-

HBc VLP is observed compared with chimeric HCV core-HBc VLP. In addition, mice immunized with both adjuvant free EBNA1-HBc and HCV core-HBc VLPs obtain long-term immunogenicity by producing epitope specific memory T cells.

In conclusion, HBc VLP platform is able to present either short and non-structural antigen or long and structural antigen to form chimeric HBc VLPs and can be effectively expressed and purified in bacteria expression system with high production yields. The produced chimeric HBc VLPs show great potentials to possess strong and long-term antigen specific immunogenicity *in vivo* without addition of adjuvant. The produced adjuvant free chimeric EBNA1-HBc VLP and HCV core-HBc VLP are promising vaccine candidates against EBV and HCV infection. In addition, the type of antigens presented by HBc VLP platform is found to influence the stability and structure of chimeric HBc VLP vaccines because of the nature and properties of the antigens. It is found that the hydrophobicity of chimeric HBc monomers is related to the stability to maintain its assembled VLP structure. Further studies of the impact of different types of epitopes on the HBc VLP assembly are still needed for the development of promising chimeric HBc VLP vaccines.

7.2 Future directions

Based on the conclusions in this thesis, chimeric HBc VLP vaccine presenting foreign epitopes targeting to oncoviruses is a promising strategy for the development of effective and safe cancer vaccines. However, the insertion of foreign epitopes to HBc VLP is found to influence the stability of chimeric HBc VLP vaccines due to the nature of the foreign epitope. To further apply HBc VLP platform in the development of cancer vaccines in the future, several investigations are needed including:

1) To further study the impact of insertion of foreign epitopes to HBc on chimeric HBc VLP stability using computational protein simulation, study on the interaction mechanism and binding energy between chimeric HBc VLP monomers is needed to explore other properties

such as surface charge and hydrogen bond that would affect the stability of chimeric HBc VLPs aiding with MD simulation,

2) Since EBNA1-HBc and HCV core-HBc has been confirmed to elicit strong *in vivo* immune response, further evaluate the virus neutralization assay for the development of produced adjuvant free chimeric EBNA1-HBc VLP and HCV core-HBc VLP vaccines.

3) It has been confirmed that HBc VLP platform can present both short and long epitopes to form chimeric HBc VLP and obtain desired immunogenicity. Further application for presenting other foreign epitopes targeting oncoviruses or other diseases caused by the virus infections using HBc VLP platform should be investigated to broaden its application not only as human vaccines but also as animal vaccines,

List of publications

[1] S. Yin, Y. Wang, **B. Zhang**, Y. Qu, Y. Liu, S. Dai, Y. Zhang, Y. Wang, J. Bi, Engineered Human Heavy-Chain Ferritin with Half-Life Extension and Tumor Targeting by PAS and RGDK Peptide Functionalization, Pharmaceutics 13(4) (2021) 521.

[2] Y. Qu, L. Wang, S. Yin, **B. Zhang**, Y. Jiao, Y. Sun, A. Middelberg, J. Bi, Stability of Engineered Ferritin Nanovaccines Investigated by Combined Molecular Simulation and Experiments, The Journal of Physical Chemistry B (2021).

[3] Y. Zhang, Y. Liu, **B. Zhang**, S. Yin, X. Li, D. Zhao, W. Wang, J. Bi, Z. Su, In vitro preparation of uniform and nucleic acid free hepatitis B core particles through an optimized disassembly-purification-reassembly process, Protein Expression and Purification (2020) 105747.

[4] H. Rastin, **B. Zhang**, A. Mazinani, K. Hassan, J. Bi, T.T. Tung, D. Losic, 3D bioprinting of cell-laden electroconductive MXene nanocomposite bioinks, Nanoscale 12(30) (2020) 16069-16080.

[5] H. Rastin, **B. Zhang**, J. Bi, K. Hassan, T.T. Tung, D. Losic, 3D printing of cell-laden electroconductive bioinks for tissue engineering applications, Journal of Materials Chemistry B 8(27) (2020) 5862-5876.

[6] Y. Zhang, S. Yin, **B. Zhang**, J. Bi, Y. Liu, Z. Su, HBc-based virus-like particle assembly from inclusion bodies using 2-methyl-2, 4-pentanediol, Process Biochemistry (2019).

[7] P. Zheng, **B. Zhang**, B. Jin, W. Guan, B. Bai, S. Dai, Synergistic Enhancement in Antibacterial Activity of Core/Shell/Shell SiO2/ZnO/Ag3PO4 Nanoparticles, ChemNanoMat 4(9) (2018) 972-981.

[8] **B. Zhang**, H. Zhang, S. Dai, J. Bi, Cell‐penetrating peptide–labelled smart polymers for enhanced gene delivery, Engineering in Life Sciences 17(2) (2017) 193-203.