Improving and Characterising Influenza and COVID-19 Vaccines

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This thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy

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Statement of original Authorship

This thesis is being presented to the University of Sydney to meet the requirements for the Doctor of Philosophy degree. To the best of my understanding and belief, the research presented in this thesis is original, except for instances specifically referenced in the text. I confirm that this material has not been submitted, either in its entirety or partially, for a degree at this university or any other institution.

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Date: 27/06/2023

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I would like to dedicate this thesis to my cherished parents, Abdullah and Bakhita, whose unwavering support and constant motivation have been the driving force behind my accomplishments. I am also deeply grateful to my beloved wife, Rehab, for her support through this journey. Additionally, I dedicate this work to my daughter Salma and my son Sultan, whose presence has brought immense joy and served as a reminder of what truly matters.

List of publications and conferences

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List of Abbreviations

APC	Antigen-Presenting Cell
CVV	Candidate Vaccine Virus
ER	Endoplasmic reticulum
HA	Hemagglutinin
IIV	Inactivated influenza vaccine
mAbs	Monoclonal antibodies
NA	Neuraminidase
NLG	N-linked glycosylation
NP	Nucleoprotein
NS2	Non-structural protein
PA	Polymerase acidic
PB	Polymerase basic
PDB	Protein Data Bank
RdRp	RNA dependent RNA polymerase
RNP	Ribonucleoprotein
SA	Sialic acid
ssRNA	Single-stranded RNA
TEM	Transmission Electron Microscopy
VLP	Virus-Like Particle
vRNP	Viral Ribonucleoprotein
LAIV	Live attenuated influenza vaccine
IIV	Inactivated influenza vaccine
LAIV	Live attenuated influenza vaccine
WIV	Whole-virus Inactivated Vaccines
CVV	Candidate vaccine viruses
APC	Antigen-Presenting Cell
DCs	Dendritic cells
UV	Ultraviolet
CMC	Critical micelle concentration
DLS	Dynamic light scattering
NR	Nile red
NTA	Nanoparticle Tracking Analysis
sCX[4]	4-Sulfocalix[4]arene
sCX[8]	4-Sulfocalix[8]arene

CB[5]	Cucurbit[5]uril
CB[7]	Cucurbit[7]uril

Thesis Abstract

The development of safe and effective vaccinations against infectious illnesses and certain cancers that cause substantial morbidity and death is a major advancement in medicine. Vaccination, as a preventative public health measure, has clearly led to better health outcomes for people all over the globe. It is believed that vaccinations have prevented six million deaths every year (1). The need of developing a strong vaccination plan for global immunisation has been reaffirmed in the light of the COVID-19 pandemic. Safe and effective vaccines can only be developed with persistent characterisation and stable formulations. In addition, efforts are made to find solutions to problems like preserving cold chains, which are essential for keeping vaccines at their peak potency and efficacy throughout storage and shipping.

The first chapter provides a general introduction to vaccinations and their background, as well as a brief synopsis of the other chapters, each of which is dedicated to one of three goals. In Chapter 2, we go deeply and exhaustively into a variety of flu-related topics (viruses and vaccines). The thesis's three aims are discussed in the following chapters. The primary objective was to find a suitable replacement for Triton X-100, which is currently used as a splitting agent in the manufacturing of split-virus influenza vaccines but is considered a "substance of very high concern" by the European Commission due to the production of harmful metabolites upon its environmental release. In Chapter 3, we see the results of an experimental study introducing an alternative to Triton X-100 that shows promise for use in the manufacturing of inactivated influenza vaccines. The second goal of this research was to conduct the first-ever evaluation of the capacity of macrocycles to stabilise influenza virus (chapter 4). Despite macrocycles' widespread use in the pharmaceutical industry, the potential of these structures to improve influenza vaccine formulations has not been investigated until recently. The third goal of this study was to investigate the effects of the stabiliser sCX[4] on the thermal stability and aggregation properties of two different COVID-19 vaccine

formulations (from Pfizer and AstraZeneca). This study set out to explain how sCX[4] affects the stability and aggregation behaviour of COVID-19 vaccine formulations.

This thesis presents novel experimental approaches that may improve and speed up the process of creating vaccines against influenza and COVID-19. These results help advance scientific knowledge and provide crucial insight for the development of future influenza and COVID-19 vaccines, which will assist public health initiatives throughout the world.

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Chapter 1

Thesis introduction

1.History

The establishment and coordination of national immunisation programs, primarily initiated in the 1960s, have had a profound impact on public health, with vaccines playing a pivotal role. In countries where vaccine programs have achieved extensive coverage, numerous diseases that once posed a substantial threat to childhood mortality have essentially been eradicated. According to the World Health Organization (WHO), the existing immunisation programs save an estimated six million lives annually, resulting in a significant decline in mortality among children under the age of five worldwide [1].

The inception of contemporary vaccination can be traced back to the groundbreaking research conducted by Edward Jenner, an esteemed English physician, during the 18th century. Jenner observed that milkmaids who contracted cowpox, a viral infection, became immune to the dreaded smallpox virus. To test his hypothesis, he deliberately inoculated the son of his gardener with pus extracted from a cowpox patient's pustule, and then subjected the child to a deliberate exposure to smallpox. Remarkably, the child successfully withstood the smallpox virus. Jenner's seminal study, published in 1798, stands as one of the most influential and transformative contributions in the annals of medical history [2]. The term "Variolae vaccinae" was coined by Edward Jenner to denote the practice of using cowpox as a means of immunisation. The word "vaccine" itself finds its origins in the Latin term "vacca," meaning cow. In accordance with modern ethical standards, Jenner's experiment would be regarded as unethical, and its methodology had various limitations. However, this study played a pivotal role in the development of the smallpox vaccine, serving as a fundamental stepping stone for the subsequent investigation and production of numerous vaccines that are widely employed today. Notably, smallpox was successfully eradicated in 1979, rendering vaccination against the disease unnecessary for children today [3].

The progress in in vitro virus culture techniques enabled a more comprehensive study of viral pathogens, while the cultivation of these pathogens under controlled laboratory conditions facilitated the development of live oral vaccines for polio, measles, rubella, mumps, and varicella viruses. During the 1960s, notable advancements took place at the Walter Reed Army Institute of Research, USA, where vaccines utilising capsular polysaccharides were created. These included vaccines for encapsulated organisms such as meningococci, pneumococci, and later Haemophilus influenzae type b (Hib) [4-6].

2- Influenza

The ability of influenza to evade our immune system is attributed to its ability to undergo extensive viral drift, characterised by minor mutations in viral genes that result in alterations of crucial surface glycoproteins such as hemagglutinin (HA) and neuraminidase (NA). Additionally, the occurrence of major structural changes in the virus, known as viral shift, can give rise to new HA and/or NA variants, often categorised as new subtypes. Furthermore, the utilization of egg-adapted virus and the consideration of the virus's selectivity, particularly for strains that do not typically circulate during a specific season. Thus, annual vaccination is necessary to effectively target the evolving influenza strains [7, 8]. A range of vaccines has been developed to address both seasonal and pandemic influenza outbreaks. These influenza vaccines undergo regular reformulation, guided by comprehensive global surveillance of the prevailing circulating strains. As a result, annual vaccination continues to be the most efficacious approach for averting seasonal epidemic influenza [8]. The current effectiveness of influenza vaccines is deemed suboptimal, with estimates suggesting a range of 40% to 60% efficacy When the antigenic properties of the vaccine strains are in alignment with those of the circulating viruses [9]. However, it is notable that the effectiveness of these vaccines has shown considerable fluctuations in recent decades, occasionally declining to as low as 10% [3]. In

order to tackle this issue, additional endeavors are warranted to enhance the stability of influenza vaccines and meticulously identify the most optimal virus candidates for incorporation into the vaccine formulation.

3- COVID-19

In 2020, the field of vaccine development faced a critical test with the emergence of a novel coronavirus, SARS-CoV-2, originating from China and leading to a widespread global outbreak of severe acute respiratory illness. The worldwide ramifications of the COVID-19 pandemic have resulted in an overwhelming number of confirmed cases, surpassing 706 million, and a devastating loss of 6.9 million lives [10]. A remarkable milestone in modern scientific progress has been the swift development and subsequent widespread distribution of vaccines targeting COVID-19. Multiple initiatives were undertaken with the goal of creating an effective vaccine against this unprecedented viral disease. Approved vaccines can be categorised into two main groups: those based on conventional platforms, such as inactivated virus vaccines, and those utilising innovative vaccine platforms, such as mRNA-based vaccines. In the aforementioned category, two primary classifications can be discerned: vaccines based on nucleic acids and vaccines based on viral vectors. mRNA-based vaccines (e.g., Pfizer/BioNTech and Moderna), adenovirus vector-based vaccines (e.g., AstraZeneca/Oxford University, Johnson & Johnson, and Sputnik V), and protein-based vaccinations are also notable examples of vaccines created utilising innovative platforms (e.g., Novavax). On the other hand, vaccines constructed using established platforms rely on inactivated virus technology, as demonstrated by vaccines such as CoronaVactm by Sinovac, Sinopharm vaccine, and Covaxin® by Bharat Biotech [3].

After the successful development of COVID-19 vaccines, vaccination campaigns were initiated across multiple nations. Pharmaceutical companies expanded their manufacturing capabilities, and large-scale vaccination centers were established. Nevertheless, the

administration of these vaccines posed significant challenges due to the intricate logistics involved, including the specific storage requirements and diverse conditions for each vaccine variant. Thorough analysis is being conducted on all vaccines, whether they are in the developmental or advanced stages, to comprehend the rapid potency degradation that occurs when exposed to temperatures above 10°C. This is crucial as the antigen component undergoes significant degradation if not kept in a frozen state. Many protein antigens are the subject of intensive research across the world right now, but even the most robust of them must be kept cold. Most established COVID-19 vaccines have strict temperature requirements for transit, ranging from 2 to 8 degrees Celsius, while some need to be frozen to a temperature of -80 degrees Celsius [11]. Hence, it is challenging for poor and middle-income nations to build a reliable cold chain infrastructure for these vaccines.

Given the existing challenges and limitations associated with the available influenza and COVID-19 vaccines, the primary objective of this thesis was to address and alleviate some of these obstacles that include improvement of influenza vaccine formulations by finding Triton X-100 alternative surfactant for split-virus influenza vaccine, enhancing the stability of whole inactivated influenza virus for novel vaccine formulations, and enhancing the stability of COVID-19 vaccines, thereby contributing to the advancement of enhanced influenza and COVID-19 vaccine development.

Thesis Overview and Chapter Descriptions

Chapter 2- An Overview of Influenza Viruses and Vaccines

Being a large body of work in the area, this review chapter offers a thorough and in-depth examination of many aspects of influenza. This chapter starts with a comprehensive look into the influenza virus and its subtypes, illuminating the differences between them and their potential consequences. Detailed information on the phases and processes of the influenza virus's life cycle is provided. Glycosylation of the hemagglutinin (HA) and neuraminidase

(NA) proteins is examined in detail to better understand their function in viral attachment and reproduction. This chapter looks further into the distinctions between seasonal and pandemic influenza to emphasise their worldwide significance. Both seasonal and pandemic influenza vaccines are discussed at length, with an emphasis on the manufacturing procedures involved. Influenza vaccinations go through extensive testing to determine what factors contribute to their effectiveness. New techniques that show promise for future influenza vaccine development are also discussed, as they were investigated in this research. Vaccine product characterisation is also extensively discussed, with the need of careful testing and inspection emphasised. Finally, the efficacy of influenza vaccines is assessed critically to provide light on the practical implications of vaccination plans. This review chapter provides researchers, clinicians, and policymakers with a wealth of information by covering all of these areas in depth, allowing them to better understand influenza and develop more effective vaccines.

Chapter 3- Triton X-100 alternative surfactant for split-virus influenza vaccine

While Triton X-100 is widely utilised as a splitting agent in the manufacturing of split-virus influenza vaccines, this study chapter tackles a crucial problem connected to this substance. Due to the production of potentially harmful metabolites when Triton X-100 is released into the environment, the European Commission has classified it as a "substance of very high concern." This chapter addresses the need for a replacement for Triton X-100 in the manufacturing of inactivated influenza vaccines by investigating the use of other surfactants.

Transmission electron microscopy, fluorescence, and light scattering are only few of the experimental methods covered in this chapter that are used to assess different surfactants. The research guarantees a thorough investigation of the discovered alternatives by concentrating on three influenza virus strains: A/California/7/2009 (H1N1), A/Switzerland/9715293/2013 (H3N2), and B/Phuket/3073/2013 (H3N2).

The potential of this research chapter to address environmental problems related to Triton X-100 without compromising the efficiency of inactivated split-virus influenza vaccines is what makes it so important. This chapter provides a candidate, Brij-56, that shows exceptional interaction skills with influenza viruses and the capacity to promote viral splitting but lacks the phenyl ether moiety.

Researchers and manufacturers of vaccines will find this chapter of great use since it contains information essential to the creation of inactivated influenza vaccines that are both safe and kind on the environment. This study adds to the advancement of vaccine production techniques and is consistent with the objective of assuring the worldwide safety of influenza vaccines by addressing the issues provided by Triton X-100.

Chapter -4 Enhancing the Stability of Whole Inactivated Influenza Virus for Novel Vaccine Formulations

In this study, the author investigates the feasibility of using macrocycles as stabilisers for influenza vaccinations. Starting out, the chapter talks about how flu vaccinations need to be more stable so that they can be more effective and last longer on the shelf. To fill this gap, we evaluate the effect of macrocycles on the conformational stability of entire inactivated influenza virus, looking specifically at the effects of 4-Sulfocalix[4]arene (sCX[4]), 4-Sulfocalix[8]arene (sCX[8]), Cucurbit[5]uril (CB[5]), and Cucurbit[7]uril (CB[7]). Methods like fluorescence, light scattering, and transmission electron microscopy are discussed in depth in this chapter as they are used to evaluate various macrocycles. The A/California/7/2009 (H1N1) and B/Phuket/3073/2013 influenza virus strains are the primary foci of the study (H3N2). The results show that sCX[4] is more effective than other examined macrocycles by a wide margin, improving conformational stability and decreasing aggregation formation. The implications of these results for the development of better influenza vaccines are substantial. This chapter of study helps to the development of influenza vaccines by finding sCX[4] as a

promising stabiliser candidate, providing prospective techniques to increase vaccine stability, hence optimising vaccination efficacy and increasing vaccine shelf life.

Chapter -5 Enhancing the Stability of COVID-19 vaccines

This study chapter looks at how the macrocycle sCX[4] affects the stability of the COVID-19 vaccines made by Pfizer-BioNTech and AstraZeneca. The structural integrity and particle characteristics of the vaccines were evaluated in the presence and absence of sCX[4] at different temperatures using a variety of analytical techniques, such as fluorescence, dynamic light scattering, static light scattering, and transmission electron microscopy. Our results show that stabilising the vaccines with sCX[4] prevents them from aggregating due to temperature changes and helps them maintain their original structures. Improving the stability of COVID-19 vaccines is important, and this study provides insight into the potential of sCX[4] and related stabilisers to do so. The results of this research may be used to improve vaccine formulations, guaranteeing their potency and quality during storage and distribution and so bolstering international efforts to prevent the COVID-19 pandemic.

Chapter -6 Conclusion

In this last section, we bring together all of the information and conclusions we've reached throughout the rest of the report. In addition, it provides helpful recommendations for additional study to advance the topic and fill up any gaps in knowledge.

Notes on the format of the chapters

Chapters 3-5 are research articles that has been arranged in the following format

1. Introduction

2. Methods

3. Results and discussion

4. Conclusion

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Chapter 2

An Overview of Influenza Viruses and Vaccines

CHAPTER 2- Authorship attribution statement

The following chapter is a full review published in the journal MDPI Vaccines

as:

R. Nuwarda[1], A. Alharbi[1], V. Kayser. (2021). An overview of influenza viruses and vaccines. Vaccines, 2021 Sep 17;9(9):1032. doi: 10.3390/vaccines9091032.
[1]These authors contributed equally as first authors to this work

I am a co-first author. I wrote the vaccines part and made number of the figures and tables.

Abdulsalam Alharbi

Signature:

Date: 27/06/2023

Permission was granted from co-authors to include the published material in this thesis.

Name: Rina Nuwarda. Signature: Date: 27/06/2023

Name: Veysel Kayser Signature: Date: 27/06/2023

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statement above is correct.

Supervisor Name: Veysel Kayser

Signature:

Date: 27/06/2023

Abstract

Influenza remains one of the major public health concerns because it causes annual epidemics and can potentially instigate a global pandemic. Numerous countermeasures, including vaccines and antiviral treatments, are in use against seasonal influenza infection; however, their effectiveness has always been discussed due to the ongoing resistance to antivirals and relatively low and unpredictable efficiency of influenza vaccines compared to other vaccines. The growing interest in vaccines as a promising approach to prevent and control influenza may provide alternative vaccine development options with potentially increased efficiency. In addition to currently available inactivated, live-attenuated, and recombinant influenza vaccines on the market, novel platforms such as virus-like particles (VLPs) and nanoparticles, and new vaccine formulations are presently being explored. These platforms provide the opportunity to design influenza vaccines with improved properties to maximize quality, efficacy, and safety. The influenza vaccine manufacturing process is also moving forward with advancements relating to egg- and cell-based productions, purification processes, and studies into the physicochemical attributes and vaccine degradation pathways. These will contribute to the design of more stable, optimized vaccine formulations guided by contemporary analytical testing methods and via the implementation of the latest advances in the field.

Keywords: influenza virus; influenza vaccine; neuraminidase; hemagglutinin; vaccine manufacturing; vaccine characterization; recombinant vaccine; universal influenza vaccine.

1. The Influenza Virus and Subtypes

The influenza virus is an enveloped virus belonging to the Orthomyxoviridae family. There are four genera of influenza virus that infect vertebrates: influenza virus A, B, C, and D which are identified by antigen differences in their matrix protein and nucleoprotein. Type A is by far the most virulent virus that causes severe respiratory disease or death. It can even lead to the emergence of a new influenza epidemic and a worldwide pandemic. Influenza B viruses also cause the seasonal flu epidemic in humans. There are two circulating influenza B lineages, B/Yamagata and B/Victoria, which are included in the seasonal flu vaccines [1]. Meanwhile, influenza C viruses commonly cause mild symptoms and are not known to cause an epidemic, and influenza D viruses have been recognized to infect swine, cattle, and sheep, and are not identified to cause disease in human [2, 3]. The virus carries a negative-sense, single-stranded RNA (ssRNA) genome that is divided into eight (subtypes A, B) or seven (subtype C, D) separate segments [4, 5].

Influenza virus A and B are indistinguishable in shape under transmission electron microscopy (TEM) (Fig. 1-1). They have spherical or filamentous forms with about 100 nm in diameter for spherical structures and frequently over 300 nm in length for filamentous shape [6]. The influenza A virion is covered with viral surface glycoproteins of hemagglutinin (HA) and neuraminidase (NA). Each virion with an average size of 120 nm has approximately 300-400 HAs and 40-50 NAs on its lipid membrane, although the number of each protein varies among different subtypes [7-9]. These two glycoproteins become the basis of further sub-division of influenza A virus. To date, 18 subtypes of HA and 11 subtypes of NA have been identified. H1-3 and N1,2 strains (e.g., as in A/H1N1 and A/H3N2) predominantly infect people and are currently circulating as seasonal influenza strains amongst humans. Influenza

infection with other subtypes such as highly pathogenic H5N1 has also been recorded to cause the outbreak in poultry and human infection [10-13].

A small number of matrix ion channel proteins (M2) across the viral membrane with a ratio of one M2 per 10^1-10^2 molecules of HA also exist [14]. The lipid envelope and its three proteins (HA, NA, and M2) coat a matrix protein called M1, which encircles the virion core. The inner part of the virion contains the non-structural protein 2 (NS2) and the ribonucleoprotein (RNP) complex, which comprises of the RNA segments layered with the nucleoprotein (NP) and RNA-dependent RNA polymerase consists of two subunits of polymerase basic (PB1 and PB2) and one subunit of polymerase acidic protein (PA).

The configuration of influenza B virion is almost identical to four membrane proteins: HA, NA, and in place of M2, NB and BM2 are found in the subtype B virus. Influenza C and D virions are structurally distinct from that of the A and B viruses, having only single primary surface glycoprotein, the HA-esterase-fusion glycoprotein, which has a similar function to HA and NA of subtype A and B viruses. Influenza C and D virus particles have a variety of shapes: they are elliptical, spherical with a diameter of 80–120 nm, or filamentous with a similar diameter but a length in the μm range [15-17].

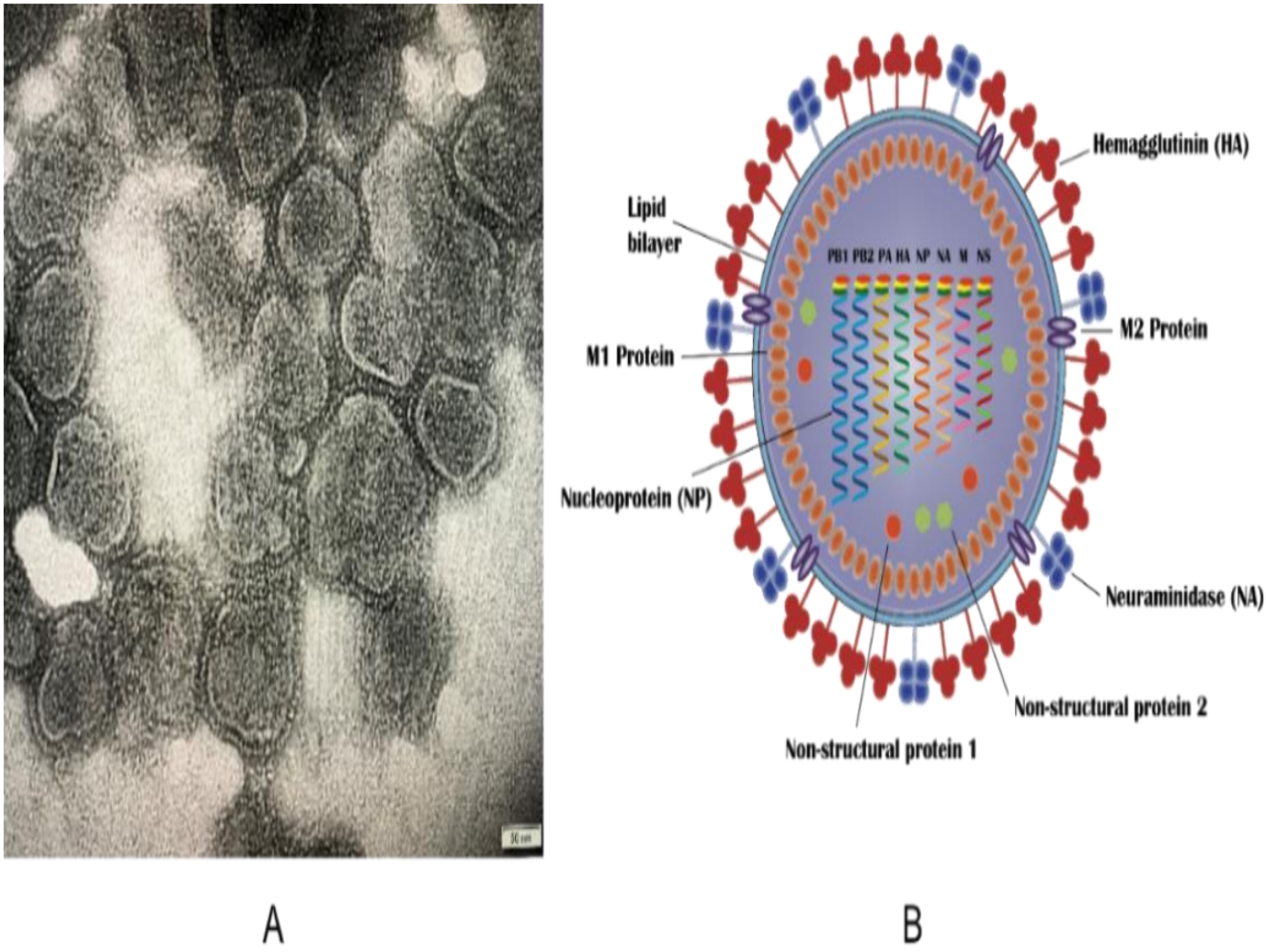

Figure 2-1. (a) TEM image of influenza virus mainly in spherical shapes with particle sizes around 100-150 nm; (b) Schematic diagram of an influenza A virus, representing the virus components, including surface glycoproteins HA and NA, M1, and M2 protein, non-structural proteins, and nucleoproteins.

2. Influenza Virus Life Cycle

The replication cycle of the influenza virus goes through several stages as shown in Fig. 2-2, and as follows: (1) Virus attachment to sialic acid receptor, (2) entry of the virus into the host cell, (3) fusion and uncoating of virus particles, (4) vRNPs entry into the nucleus followed by transcription and replication of the viral RNA genome then export of vRNPs from the nucleus,

(5) assembly of viral components and budding at the host cell membrane, and finally (6) the release of new virions from the host cells.

(1) Virus attachment to sialic acid receptor: the first stage of viral infection is the attachment of HA to the sialic acids located at the host cell membrane's surface. Sialic acids are connected to the carbohydrates of HA via glycosidic linkage. There are two linkages which are essential for the specificity of HA; (i) α (2, 3) linkage which is abundant in the digestive tract in avian or in the bronchial tissue in human, monkeys, horses, as well as upper respiratory tract of the lung epithelium in swine, and (ii) α (2, 6) which is found on the cell surface of the human upper respiratory tract as well as the trachea of bats and swine [4, 18]. (2) Entry of the virus into the host cell: receptor-mediated endocytosis occurs upon virus binding, and the virus enters the host cell in an endosome. (3) Fusion and uncoating of virus particles: The acidic pH of the endosome (pH 5-6) causes the fusion of the viral and endosomal membranes and opens up the M2 ion channel protein and acidifies the nucleus, thus allowing vRNP to be released from M1 to enter the host cell's cytoplasm and then to the nucleus.

(4) vRNPs entry into the nucleus followed by transcription and replication: the viral proteins that constitute the vRNP (NP, PA, PB1, and PB2) detect the nuclear localization signals which can attach to the cellular nuclear import machinery and consequently, enter the nucleus to undergo transcription and replication processes. The negative-sense RNA is first transformed into a positive sense RNA and serves as a template for the generation of viral RNA, followed by the internal RNA synthesis initiated by the viral RNA-dependent RNA polymerase (RdRp). Through its C-terminal domain, the RdRp associates with the large subunit of RNA Polymerase II (Pol II), which continue the transcription to produce mature mRNA. vRNPs are then exported out from the viral core through the nuclear pores [19, 20].

(5) Assembly of virus components and budding: the virus' protein components, i.e., HA, NA, and M2, are transported to the membrane's apical region where the virions bud from the polarized epithelial cells. (6) Release of new virions from the host cells: after forming viral particles, the sialic acid residues from glycoproteins and glycolipids are cleaved by NA, enabling the newly synthesized viral particles to be released from the host membrane and spread to the nearby cells [21].

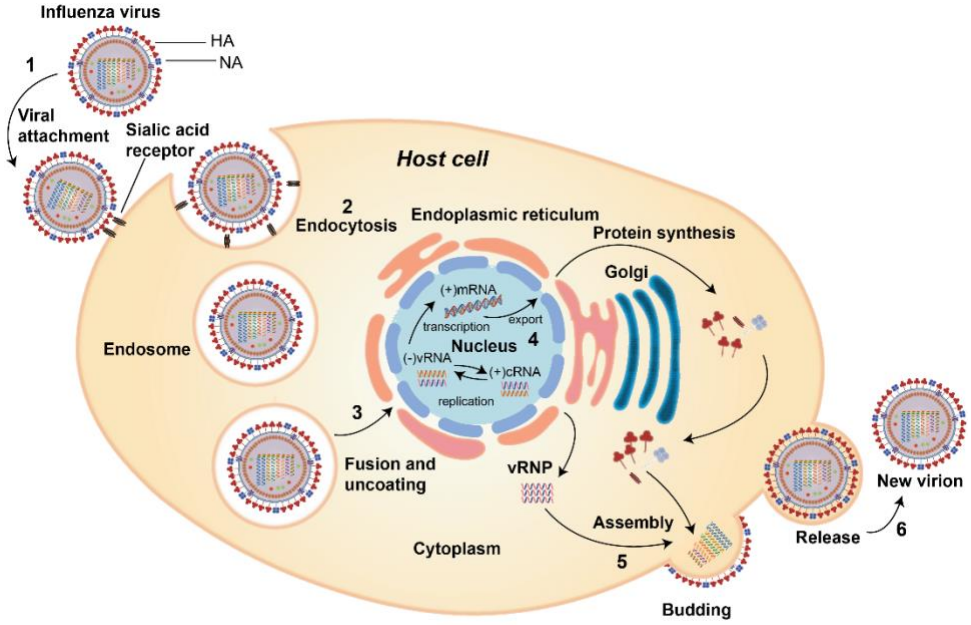

Figure 2-2. The life cycle of influenza A virus: (1) Virus attachment to sialic acid receptor via HA; (2) entry of the virus into the host cell via endocytosis; (3) fusion and uncoating of virus particle; (4) vRNPs entry into the nucleus followed by transcription and replication of the viral RNA genome and then export of vRNPs from the nucleus; (5) assembly of viral components and budding at the host cell membrane; (6) new virion release from the host cell.

3. HA and NA of Influenza Virus

HA and NA are the major transmembrane glycoproteins, with HA representing roughly 80% of the virus surface. These two glycoproteins recognize the same molecule of the host

cell, sialic acid (SA) [22]. In addition to the virus' infectivity and transmissibility, these two proteins are also associated with virulence properties, host specificity, and resistance to antiviral treatment of the virus [7]. The ribbon diagram of HA and NA structures is shown in Fig. 2-3.

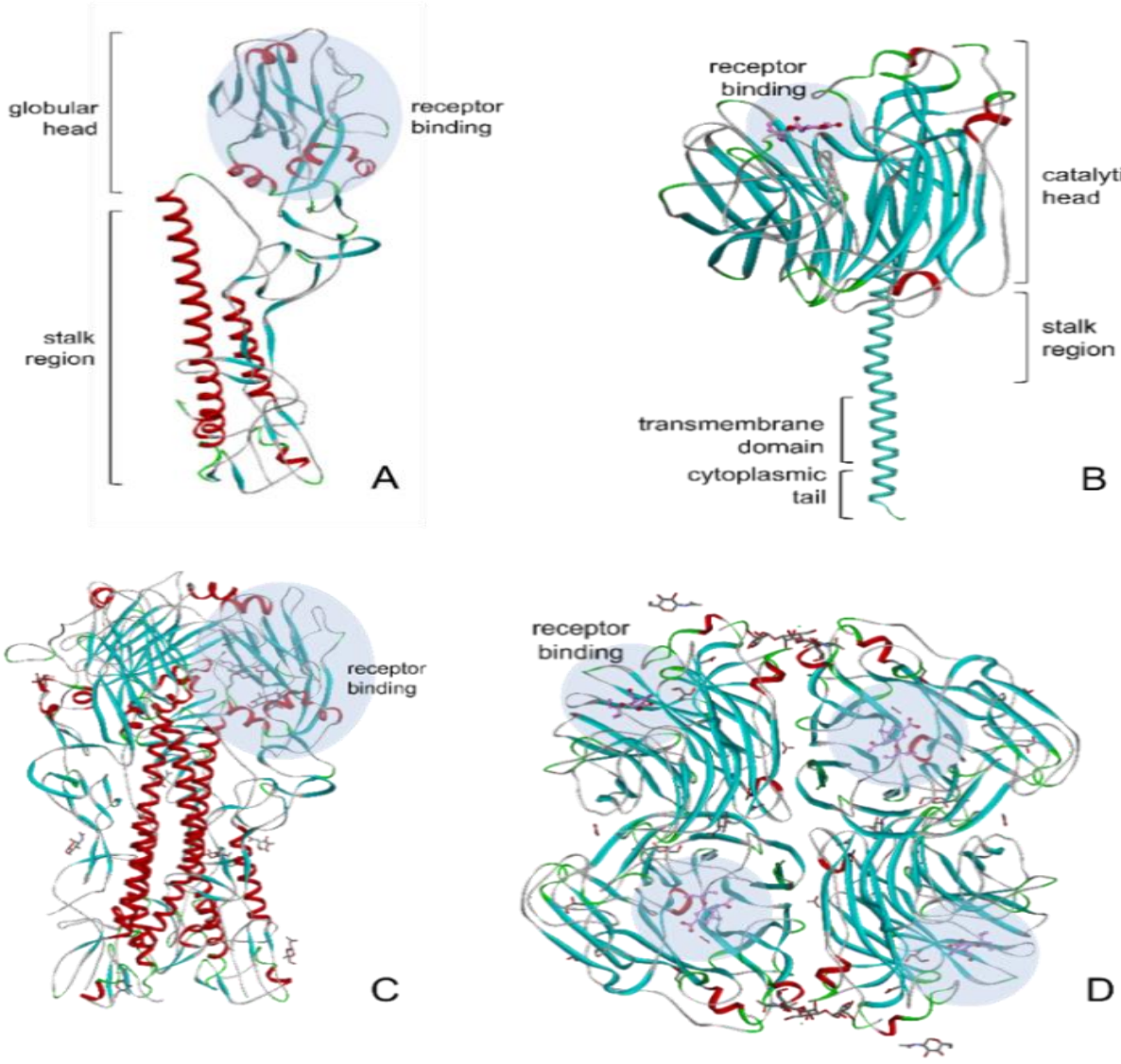

Figure 2-3. Ribbon diagrams of the 1918-human HA (Protein Data Bank (PDB) ID: 1RUZ) and 2009 H1N1 NA in complexes with oseltamivir (PDB ID: 3TI6) with their receptor binding sites shown in blue circles. (a) HA monomer; (b) NA monomer; (c) trimeric HA and (d) tetrameric NA shown from the top.

HA comprises three indistinguishable subunits (homotrimer) attached to the viral lipid membrane with each monomer possessing a globular head containing the receptor-binding domain, and a stalk region (Fig. 2-3A) [23]. This protein is responsible for the initial contact between the virus and its target cell containing terminal sialic acid residues. Moreover, HA contributes to the virus' internalization with the host cell through endocytosis, thus delivering the nucleoprotein into the cytoplasm. HA acts as the major antigen on the flu virus and becomes the primary target for antibody neutralization [24].

The NA (Fig. 2-3.B) is a tetrameric protein with four identical polypeptides consisting of a globular head, a cytoplasmic tail, a transmembrane domain, and a stalk region [25]. It is an enzyme that destroys the receptors recognized by HA by catalyzing the cleavage of terminal α-(2,3 or 2,6)-ketosidic linkage sialic acid from a range of glycoconjugates such as glycoproteins and glycolipids, therefore playing an essential role in the replication cycle of the virus. NA acts as a biological scissor that cleaves sialic acid residues from HA and cell surface glycans and facilitates the movement of the virus during virus entry, as well as from the surface glycoprotein of newly synthesized virions, hence allowing the virus to be released from the host cell and carry on the infection to other cells [26].

Influenza viruses are completely reliant on a well-balanced action of HA and NA to establish a productive infection. During initial infection, an increase in receptor-binding affinity appears to necessitate an increase in the viral NA's receptor-destroying activity as well. On the other hand, enhanced receptor binding is a serious disadvantage in the late stage of infection because it prevents new virions from being released from host cells [27].

4. Glycosylation of HA and NA

During the replication cycle of the influenza virus, small changes in virus' genes via mutations might occur due to the error-prone replication process resulting in antigenic drift[1]. Aside from the alteration in amino acid sequences, antigenic drift often involves the changes in glycosylation patterns of viral glycoproteins. HA and NA both possess potential regions for N-linked glycosylation, the addition of oligosaccharide chains through N-glycosidic bonds to the Asn residues which exists as part of the Asn-AA-Ser/Thr sequence, where AA can represent all amino acids except for proline. The attachment happens with the aid of glycosyltransferases and oligosaccharyltransferase enzymes located in the endoplasmic reticulum (ER), followed by the structural alteration of the oligosaccharides by mannosidases and glycosidases during transfer through ER and Golgi bodies [28]. This post-translational modification is vital for the influenza virus life cycle by affecting the biosynthesis, structural stability, and glycoproteins' function.

Glycosylation sites of HA are located in the stalk region and contribute to the molecular stability of HA, its receptor binding and the immune response of the host [29]. Prior studies have also suggested that glycosylation on the HA globular head domain may expose or protect the antigenic sites, leading to virus evading neutralizing antibodies [30-34].

Four seasonal human influenza strains, two A subtypes (A/H1N1 and A/H3N2) and two B lineages (B/Victoria and B/Yamagata) have circulated globally since 1977 [35, 36]. Fig. 2-4 demonstrates the evolvement of the glycosylation patterns of A/H1N1 and A/H3N2 strains from 1918 to 2018, i.e., when they were first introduced to humans and those currently circulating. The glycosylation in the stalk region of HA of 1918 pandemic A/H1N1 and 1968

A/H3N2 influenza viruses were known to be highly conserved. The 1918 A/H1N1 possessed six conserved N-linked Glycosylation (NLG) sites at residues 27, 28, 40, 304, 498, and 557 (Fig. 2-4.A), while 1968 A/H3N2 had five glycosylation sites at residues 24, 38, 54, 301, and 499 (Fig. 2-4.B). The globular head of HA, on the other hand, had gone through subsequent glycan gains and losses over the years. The number of NLG sites in the A/H3N2 HA globular head has increased from initially two (at residues 97 and 181) in 1968 to eight (at residues 79, 138, 142, 149, 160, 174, 181, and 262) on the current 2018 seasonal strains after the disappearance of site 97 around 1975. Similarly, 1918 A/H1N1 acquired an increasing number of glycans until its disappearance in 1957 and had the same glycosylation profile when it re-emerged in 1977 until the 2009 A/H1N1 strain replaced it. The current circulation A/H1N1 strain is the result of reassortment of swine lineage viruses and the glycosylation is similar to the very early human A/H1N1. Meanwhile, the NLG sites for the influenza B virus have been relatively stable at around 10-12 sites since the splitting of Victoria and Yamagata lineages in 1983 [28].

Changes in glycosylation sites and glycan profiles in HA affects how viruses interact with the host immune system. The evasion from neutralizing antibody recognition enables the virus to continue circulating in the human population. Consequently, more studies regarding the glycosylation pattern of viral glycoprotein are needed in designing better vaccines against influenza viruses [34, 37].

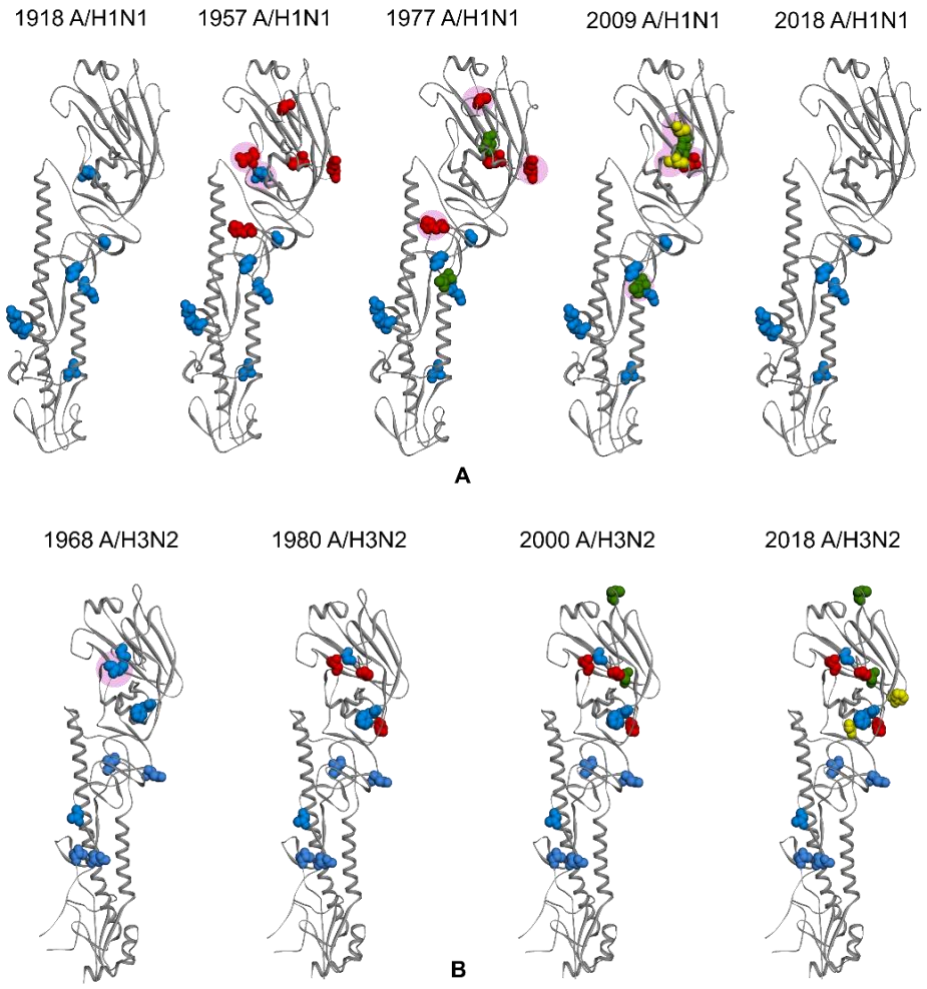

Figure 2-4. Some of the NLG sites of 1918 HA (A) and 1968 HA (B). Blue color residues represented the NLG sites when they were first identified, while red, green, and yellow color residues represent NLG sites acquired afterward. The pink circle represents the NLG sites that have been lost during the evolution. 1918 A/H1N1 initially possessed six NLG sites and acquired five more in 1957. 1977 A/H1N1 gained two NLG sites but lost two, whereas 2009 A/H1N1 gained two sites and lost three. The current 2018 A/H1N1 circulating strain have lost five more NLG sites compared to 2009 strain. Meanwhile, NLG sites in 1968 A/H3N2 HA globular head has been gradually increasing from initially two in 1968 to eight in the current 2018 strain (with the loss of site 97 in 1975).

While the NLG of HA known to contribute to immune escape and influenza viruses' virulence, it is still unclear how the NLG of NA affects its function. Previously published research established that glycosylation of the N8 NA protein is necessary for proper tetramerization [28]. Additionally, glycosylation of the head domain of the NA protein is required for proper maturation [39]. In recent study, Bao et al., explored the function of the NLG modification in the NA protein of the A/1933/WSN/H1N1 (WSN) virus. The results indicated that the NLG modification of NA protein was critical for its budding. In transfected cells, non-glycosylated NA protein lost its ability to bud. Additionally, the absence of the NLG modification significantly reduced the mutant virus's budding and replication. The deletion of NLG at the 219 position significantly attenuated the WSN virus in a mouse model. It was suggested that overexpression of non-glycosylated NA protein in cells induced the unfolded protein response (UPR), which may affect the ability of NA proteins to budding, thereby attenuating the mutant virus's virulence [40].

5. The Influenza Epidemic vs. Pandemic

Influenza viruses can cause severe contagious disease by infecting the respiratory tract and may result in hospitalization and even death. Annual seasonal epidemics of influenza were predicted to have approximately 3 to 5 million cases of severe illnesses and 290,000 to 650,000 related respiratory deaths globally [41]. The highest mortality rates were estimated in Southeast Asia and sub-Saharan Africa, and high-risk groups of severe outcome and death include people aged 75 years or older, children younger than five years, and individuals with lingering illnesses that put them at more significant risk of acquiring influenza or its complications [41, 42].

New strains of influenza A virus appear sporadically every 2 to 5 years as epidemics and last between 3 and 6 months depending on the region. The influenza epidemic is often referred to as the flu season, which is potentially tied to the weather and climate. The introduction of specific mutations within surface glycoproteins HA and NA enables the virus to evade the host immune system and results in no permanent immunity against the virus after neither natural infection nor vaccination. The minor changes of the influenza virus' antigenicity are described as antigenic drift and become the basis for the influenza epidemic's frequent occurrence, making it necessary to update influenza vaccines annually to match the currently circulating strains subjected to WHO recommendation [43-45].

Influenza pandemics, on the other hand, result from significant changes in the virus's antigenicity and are suggested to happen approximately every 10 to 40 years [46]; however, it could happen anytime. History documented that no less than three pandemics occurred during the twentieth century: the 1918-19 Spanish flu, 1957-58 Asian Flu and 1968-69 Hong Kong Flu, with respective mortality rates varied from devastating to mild [44]. These pandemic viruses obtained new subtypes by reassortment of RNA segments from distinct influenza viral strains. This genetic change, referred to as an antigenic shift[2], permits a viral strain to jump from one species of animal to another and from animals to human [48, 49].

The 1918 pandemic, also termed as Spanish Flu, was the first and remains the most disastrous influenza pandemic in history. It was estimated that the virus spread worldwide by infecting about 25-30% of people worldwide and killing approximately 50 million individuals, around 2.7% of the world's population [50, 51]. A more recent analysis by Spreeuwenberg et al. in 2018 indicated that earlier estimations were excessively high. Their own estimate puts

the death toll at 17.4 million, 0.95% of the world's population [52]. 1918 pandemic was caused by the A/H1N1-subtype influenza virus, which was considered as the common ancestor of nearly all human influenza A cases. These include not only antigenically "drifted" descendants of the 1918 H1N1 virus, but also genetically reassorted pandemic viruses that emerged in 1957 (H2N2), 1968 (H3N2), and 2009 (H1N1). [53]. The H2N2 subtype of influenza A virus which emerged in Hunan Province of China in February 1957, believed to be clinically milder than the H1N1 virus that caused the 1918 pandemic with a mortality rate worldwide of 1.1 million deaths [54]. The virus circulated globally until H3N2 appeared in 1968, causing another pandemic. It first emerged in China, then spread rapidly to Hong Kong. The 1968 pandemic, also called Hong Kong flu, was comparatively a milder pandemic with the death toll around 1 million people [55, 56]. The influenza pandemic in the twenty-first century started in early 2009 when a novel swine-origin flu virus appeared and substituted the former seasonal influenza H1N1. Initially identified in Mexico, the virus spread rapidly worldwide and was declared a pandemic by WHO in June 2009 after 74 countries reported the infections [57, 58]. The virus, also called "2009 H1N1", was a reassortment product between two pre-existing swine flu viruses—a North American H1N2 and a Eurasian H1N1 [59].

There has been an increase in reports of direct bird-to-human transmission of avian influenza viruses in the last two decades, resulting in an ongoing outbreak of influenza A/H5N1 among poultry with associated human infections. In 1997, fatal cases of H5N1 infections in humans were first detected in Hong Kong. Six years later in 2003, the virus re-emerged as a highly pathogenic avian influenza virus and caused devastating outbursts in local poultry, raising concerns about a potential influenza pandemic [60-62]. The outbreaks had become endemic in several countries, with the highest number of cases occurring in Egypt, Indonesia, and Vietnam [63]. In 2007, there was evidence of limited person-to-person transmission of the

H5N1 in China. Furthermore, WHO reported evidence of non-sustained human-to-human H5N1 virus spread in Indonesia and Pakistan in 2006 and 2007, respectively. The first case of H5N1 infection in the Americas was reported in Canada on January 8, 2014, in a traveler returning from China. Although human infections with this virus are uncommon, approximately 60% of cases result in death [64]. In November 2019, the WHO declared the total number of 861 verified human cases for avian influenza A (H5N1), leading to 455 death cases since 2003, with one last case reported in Nepal on 30 April 2019 [65].

6. Vaccines Against Influenza Epidemic and Pandemic

Several vaccines exist to combat the influenza epidemics and pandemics. Influenza vaccines are reformulated each year based on global surveillance of the current circulating strain. Therefore, annual vaccination remains the most effective way to prevent seasonal epidemic influenza.

Currently, there are three types of vaccines used worldwide: inactivated influenza vaccine (IIV), live attenuated influenza vaccine (LAIV), and recombinant HA vaccine (Table 1). Each type of vaccine has its advantages and disadvantages. Vaccines can induce immune effectors, including humoral effectors (antibodies produced by B lymphocyte), that can bind specifically to the virus antigen, and cellular effectors (cytotoxic CD8+ T lymphocytes) that can limit the spread of the virus inside the body by killing the infected cells. All influenza vaccines must be annually updated because the immunity only lasts for a short period of time [62] and to matches the antigenicity of the circulating viruses [63, 64]. Hence, WHO and other stakeholders meet biannually to select the most appropriate influenza viruses from globally circulating viruses according to their antigenic and genetic characteristics and the epidemiologic information from different countries. One of these influenza vaccine composition meetings occurs for the Northern hemisphere and the other for the Southern hemisphere [65, 66].

Trivalent vaccines containing two influenza (H1N1 and H3N2) and one lineage influenza B viruses were formulated to provide protection against three distinct influenza viruses for many years, even though there are two lineages of circulating B viruses. Therefore, in order to give wider protection against the current circulating viruses, a second lineage of B virus was added to the formulation for a quadrivalent influenza vaccine. Current approved influenza vaccines are quantitatively standardized in terms of HA quantity or antigenicity, but not in terms of neutralizing antibodies (NA). As a result, both the NA content and the NA immunogenicity of vaccinations may be very varied.

6.1. Inactivated Influenza Vaccine (IIV)

The inactivated virus vaccine is the most common worldwide approach due to its high safety and relatively low production costs. As a result, it has the highest percentage in the flu vaccine global market. The IIV can be given for children six months and older like Afluria Quadrivalent™, Fluarix Quadrivalent™, FluLaval Quadrivalent™, and Fluzone Quadrivalent™; however, some IIVs are only for individuals who 65 years and older, like Fluad™ and Fluzone High-dose Quadrivalent® (Table 1). The virus is generally grown in embryonated chicken eggs or cultured cells of mammalian origin for this type of vaccine (Fig. 2-5). It has been shown that IIV can induce local and systemic immunity, although booster vaccinations might be needed to maintain the antibody titers [67]. IIV has three types: whole-virus inactivated vaccines, split-virus inactivated vaccines, and subunit inactivated vaccines.

6.1.1. Whole-virus Inactivated Vaccines (WIV)

The whole-virus vaccine was the first of the influenza vaccines to be used. However, a number of systemic and local side effects have been reported upon administration. These adverse effects could result from the impurities in these vaccines, such as egg proteins [68].

However, improved technologies in vaccine production led to safer vaccines with lower impurities and fewer side effects [69]. In WIV, after the incubation and growth in embryonated eggs, virions are chemically inactivated mainly with formaldehyde or β-propiolactone, then purified (Fig. 2-5). Although this method is the most used approach for influenza virus inactivation, other methods can be used for virus inactivation, including heat and radiation [70].

The use of this type of influenza vaccine is currently limited due to the other types that entered to the market, such as split-virus inactivated vaccines, which have fewer reactogenic effects. 3Fluart™ is a WIV produced by Fluart Innovative Vaccines Kft. and is used only in Europe.

6.1.2. Split-virus Inactivated Vaccines

Majority of the currently used influenza vaccines are split-virus or subunit vaccines due to their adequate immunogenicity and ease of production. However, they lose some of the inherent immunogenicity because of the lack of the whole virus structure unlike WIV [69]. The split-virus vaccine can be prepared by disrupting the virus membrane chemically using surfactants such as Triton-100 or Octyl glycoside. Then, the surfactant is removed by tangential flow filtration [71]. The seasonal influenza split-virus vaccines in the US (representing the northern hemisphere) and Australia (representing the southern hemisphere) are summarized in Table 1 with their manufacturer and age groups information. In Australia, most of the seasonal influenza vaccine are split-virus inactivated vaccines with five vaccines in the market (FluQuadri®, Vaxigrip Tetra®, Fluarix Tetra™, Afluria QuadrivalentTM, Influvac Tetra™). In US, also split-virus inactivated vaccines have the dominant part of seasonal influenza vaccines with five vaccines (Afluria Quadrivalent™, Fluarix Quadrivalent®, FluLaval Quadrivalent™, Fluzone Highdose Quadrivalent®, Fluzone Quadrivalent®).

6.1.3. Subunit Inactivated Vaccines

Generally, subunit vaccines contain only the antigenic parts of the virus: HA and NA. These proteins are either purified from the influenza virus after splitting the virus using surfactants (Fig. 2-5). This type of influenza vaccine usually needs an adjuvant to induce an adequate immunogenicity, especially in the elderly [72].

Fluad® and Fluad Quadrivalent® are subunit influenza vaccines by Seqirus that can be given to 65 years and over. Another vaccine, Influvac Tetra™ manufactured by Mylan Health, is used for three years and older (Table 1). Due to their comparable immunogenicity and low reactogenicity, split-virus and subunit-virus vaccines have been more commonly used than whole-virus vaccines since the 1970s [73].

6.2. Live attenuated Influenza Vaccines (LAIV)

The LAIV was developed to mimic natural infection and immunity without causing severe illness and consequently induces humoral and cellular immunity. LAIV was first proposed back in the 1960s by growing the influenza viruses under suboptimal conditions in eggs [74].

These attenuated viruses are temperature-sensitive and grow only at 25°C (cold-adapted). Cold-adapted donor viruses undergo several passages with a gradual reduction in the temperature in embryonated chicken eggs. Thus, LAIV can grow at the same temperature range as the nasopharynx's mucosal surface when LAIV is administered intranasally [75]. LAIV has some superiority over IIV because it induces mainly local mucosal immunity and local immunoglobulin A (IgA) production as well as IgG [67, 75, 76], in contrast to only systemic IgA and IgG antibodies with IIV. However, due to the risk of using live viruses for

immunization, LAIV is not recommended for immunocompromised individuals with low immunity (immunocompromised) or people who are in close contact with them.

FluMist Quadrivalent^{TM} (Table 1), which is manufactured by MedImmune and approved in the US, is an intranasal LAIV seasonal influenza vaccine that can be used safely in children and adults, ages 2 to 49 years, and contain four influenza strains (two influenza A and two influenza B). [77-79]. Fluenz TetraTM, manufactured by AstraZeneca and approved in Europe, is another LAIV used for ages from 2 to 17 years. No LAIV is approved in Australia as of 2021.

6.3. Recombinant HA Vaccine

Recombinant HA vaccine can be produced using recombinant protein expression technology by insect cells and baculovirus due to their high yield and cost effectiveness (Fig. 2-5) [80]. Insect cells are the typical cell lines used for recombinant protein expression, and Gibco® Sf9 cell line is the most commonly used in human or veterinary medicine for recombinant protein expression [81].

The recombinant HA vaccine has an advantage over egg-based influenza vaccines: there are no unwanted mutations from egg adaptation (Table 1). Moreover, recombinant HA vaccine is an appropriate option for people with egg allergy [82]. Although recombinant HA vaccine and IIV have a similar mechanism of action, recombinant HA vaccine is less immunogenic and hence requires three times more HA than IIV in order to induce the same antibody titers as in IIV [83]. Another advantage of the recombinant HA vaccine is its suitability for an influenza pandemic because it might take shorter time to manufacture and it would be much safer than other influenza vaccines due to the absence of highly pathogenic viruses [84]. However, due

to its lack of efficacy in children, recombinant HA vaccine is limited to adults [85] [64]. Flublok Quadrivalent®, manufactured by Sanofi Pasteur, is the first recombinant HA vaccine (Table 1).

Table 1. Various licensed seasonal influenza vaccines available in the US (representing the northern hemisphere) and Australia (representing the southern hemisphere).

No	Tradename	Manufacturer	Age Group	Country	References
Inactivated Influenza Vaccine, Split virion, egg-based					
1	Afluria Quadrivalent	Seqirus Pty. Ltd.	Six months and over	US	[86]
2	Fluarix Quadrivalent	GlaxoSmithKline Biologicals	Six months and over	US	[87]
3	FluLaval Quadrivalent	ID Biomedical Corporation of Quebec	Six months and over	US	[88]
4	Fluzone Highdose Quadrivalent	Sanofi Pasteur, Inc.	65 years and over	US	[89]
5	Fluzone Quadrivalent	Sanofi Pasteur, Inc.	Six months and over	US	[90]
6	FluQuadri	Sanofi-Aventis Australia	Six months and over	Australia	[91]
7	Vaxigrip Tetra	Sanofi-Aventis Australia	Six months and over	Australia	[92]
8	Fluarix Tetra	GlaxoSmithKline Biologicals	Six months and over	Australia	[88]
9	Afluria Quadrivalent	Seqirus Pty. Ltd.	Five years and over	Australia	[93]
10	Influvac Tetra	Mylan Health	Three years and over	Australia	[93]
Inactivated Influenza Vaccine, Surface antigen, adjuvanted, egg-based					

11	Fluad	Seqirus, Inc.	65 years and over	US	[94]
12	Fluad Quadrivalent	Seqirus, Inc.	65 years and over	US	[95]
13	Fluad Quad	Seqirus, Inc.	65 years and over	Australia	[94]
Recombinant vaccine					
14	Flublok Quadrivalent	Sanofi Pasteur, Inc.	18 years and over	US	[96]
Inactivated subunit Influenza Vaccine, cell culture-based					
15	Flucelvax Quadrivalent	Seqirus, Inc.	Four years and over	US	[97]
Live Attenuated Influenza Vaccine					
16	FluMist Quadrivalent	MedImmune, LLC	Two years through 49 years	US	[98]

7. Influenza Vaccine Manufacturing Processes

At present, influenza vaccines can be produced in three different approaches: conventional egg-based vaccines (the traditional method), cell-based vaccines, and recombinant HA vaccines. These methods are summarized in Fig. 2-5. Both egg-based and cell-based vaccine manufacturing begins with candidate vaccine viruses (CVV) grown in eggs or cells (Step 1). After harvesting, separation, filtration, and purification (Step 2), the virus is inactivated chemically generally using formalin to make inactivated influenza vaccines or disrupted chemically by surfactants to prepare split virus vaccines or subunit vaccines (Step 3). The recombinant influenza vaccine does not need growing the whole virus; only the genetic information of the HA antigen is obtained and combined with a baculovirus to facilitate delivering the genetic information (DNA) into a host cell for expressing the HA antigen. The

final step involves fill-finish processes and packaging, delivering, and storage of the product under cold-chain conditions (Step 4).

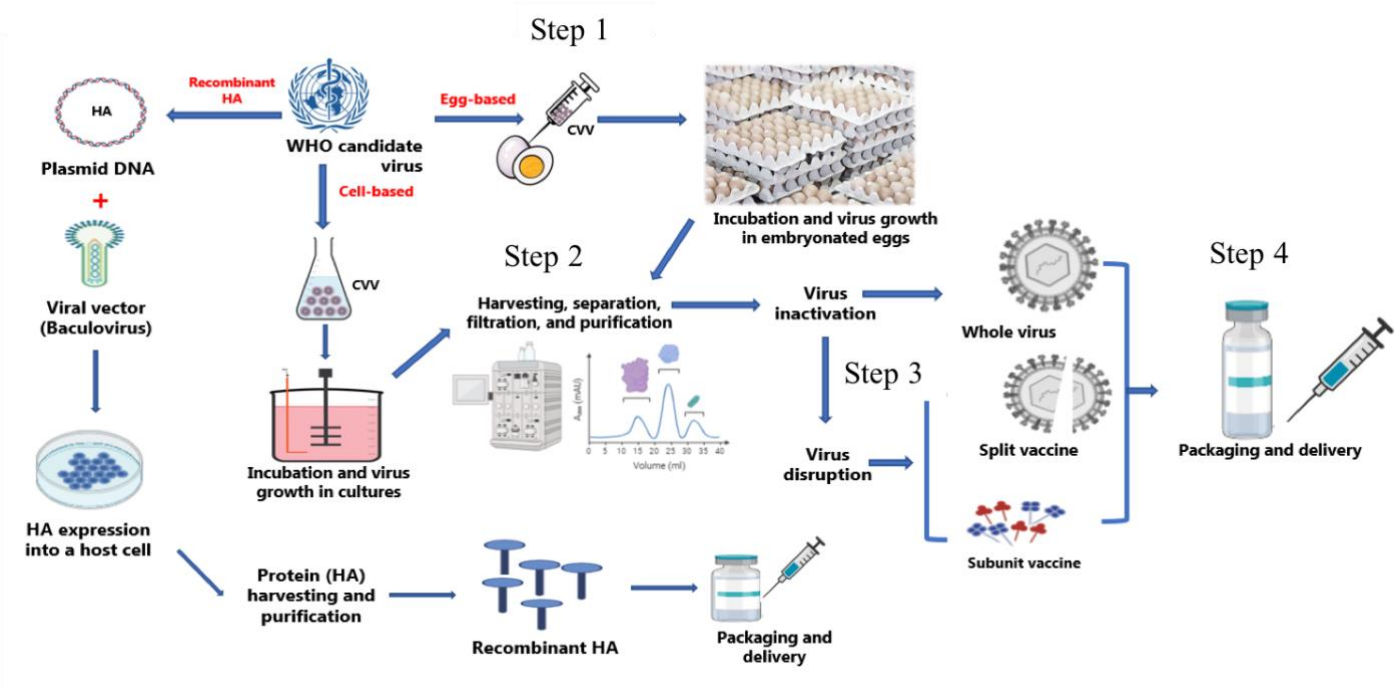

Figure 2-5. Influenza vaccine manufacturing processes. Both egg-based and cell-based vaccine manufacturing begins with candidate vaccine viruses (CVV) grown in eggs or cells (Step 1). After harvesting, separation, filtration, and purification (Step 2), the virus is inactivated chemically generally using formalin to make inactivated influenza vaccines or disrupted chemically by surfactants to prepare split virus vaccines or subunit vaccines (Step 3). The final step involves fill-finish processes and packaging, delivering, and storage of the product under cold-chain conditions (Step 4). The recombinant influenza vaccine does not need growing the whole virus; only the genetic information of the HA antigen is obtained and combined with a baculovirus to facilitate delivering the genetic information (DNA) into a host

cell for expressing the HA antigen. This antigen is produced, harvested, filtered, purified, and then vaccine is prepared.

7.1. Egg-based Vaccines

Since the 1940s, egg-based vaccines have been the most common method for producing millions of influenza vaccines annually [99, 100]. An abundant egg supply is needed to grow target viruses in chicken eggs regardless of vaccine strain [101]. This method starts with the selection of the predicted circulating virus strains, followed by the reassortment of the genetic segments of HA and NA in an egg-adapted virus that contains other gene segments from strains that can grow in the eggs in large amounts with a good safety profile like A/Puerto Rico/8/1934 [85]. After these reassorted viruses are generated, they are injected in embryonated chicken eggs to grow and then sequenced for confirmation to collect the candidate virus. The approved virus is then delivered by centers affiliated with WHO Global Influenza Surveillance and Response System (GISRS) to private manufactures [102]. The manufacturers mass-produce the virus in embryonated chicken eggs, then purify the reassorted viruses. Then, the purified viruses are chemically inactivated using formalin or β-propiolactone, and the content of HA is standardized by removing the contaminants of nonviral proteins [103]. The HA protein is the key factor for the induction of antibodies against influenza viruses and, consequently, is the focus of influenza vaccine development [104, 105].

Although the egg-based manufacturing method has many benefits, such as obtaining generally a high titer of influenza virus and significant experience in large-scale production, several drawbacks can affect its efficiency. Firstly, the adaptation of the reassortant viruses in eggs and screening the antigenicity of isolate viruses delay the time needed for the production of influenza vaccines for the next influenza season and, consequently, necessitate the start of

production of influenza vaccines long before the upcoming influenza season [106, 107]. Some influenza strains (like H3N2 viruses), however, are poorly grown in eggs [108]. As a result, the production of influenza vaccines with such strains takes a long time and delay the delivery of the vaccines by affecting the standard production time frame [109]. During egg-based manufacturing, viruses are not monitored and mutate. This egg adaptation can lead to the production of viruses that poorly match the circulating strains, which contributes to the decrease in vaccine efficacy [108, 110]. Additionally, the threat of newly discovered flu viruses emerging as well as contamination in poultry houses while egg production has raised bio security concerns during the egg-based vaccine manufacturing process.

7.2. Cell-based Vaccines

The FDA announced the approval of Flucelvax® in 2012, the first approved cell-based influenza vaccine in the US. Madin-Darby Canine Kidney (MDCK) cells are used to grow viruses [111]. Reassortment of viruses in the cell-based method can be generated similar to the egg-based method using HA and NA of selected strains [100]. The cell-based approach has some advantages over the egg-based method. Firstly, using cells can overcome the limitation of egg-shortage and offers a more flexible vaccine production approach due to the dependence on the bioreactor's capacity. Thus, cell-based vaccines can be produced in less time compared to egg-based ones [100]. Moreover, a more heterogeneous glycosylation profile of HA protein can be observed when growing the virus in egg [112]. Some studies investigate the theory that using mammalian cells as a substrate can enhance the vaccines' immunogenicity compared to avian cells [113].

Furthermore, cell-based vaccines are a suitable alternative for people who have previously experienced egg allergy, as their products are free from egg protein. Interestingly, one of the

most important advantages of the cell-based method is the lack of HA mutations due to egg-adaptation. Thus, in 2016, the FDA has approved the use of cell-based virus to prevent further mutations caused by egg-adaptation [114].

Although advantages of the cell-based method exist, some drawbacks remain. The experience in the large-scale production of cell-based vaccines is still less than egg-based vaccines. Moreover, according to the CDC, Flucelvax® can cost 40% higher than egg-based vaccines. Albeit to a lesser degree, mutations can still happen in HA and NA proteins in cell culture due to serial passaging [115].

8. Influenza Vaccine Formulation and Ingredients

Vaccines must contain ingredients that are confirmed to be safe for humans. In addition, each vaccine component serves a specific purpose, i.e., increasing the immunogenicity of antigens and keeping the vaccine formulation stable.

8.1. Active Components

The viral antigens, the active pharmaceutical ingredient (API), serve as the immunogenic components that is the most crucial part of the vaccine. Every influenza season, WHO has recommended four influenza viruses to be included in quadrivalent vaccines and three strains in trivalent vaccines. Thus, quadrivalent influenza vaccines contain four influenza strains (two influenza A strains and two influenza B strains), and trivalent influenza vaccines contain three influenza strains (two influenza A strains and one influenza B strain).

8.2. Adjuvants

Adjuvants are compounds that improve the immune reaction to a vaccine. Aluminum salts such as aluminum hydroxide (Panflu^{TM}), aluminum phosphate, and potassium aluminum sulfate are among them. Adjuvants are believed to increase immune response by having the antigen(s) close to the injection site, where immune system cells can easily reach them [116]. The usage of aluminum adjuvants in vaccines usually results in less antigen per dose of vaccine and, in certain instances, requires fewer vaccine doses. The involvement of adjuvants in vaccines is often linked to local reactions at the injection site following vaccination [117]. Other adjuvants can be used in flu vaccines such as virosomes or oil-in-water emulsions (MF59, AS03, AF03). Fluad Quad^{TM} is the only adjuvanted (MF59) flu vaccine in Australia.

8.3. Stabilizers

Stabilizers are used to preserve a vaccine's potency by maintaining the antigen and other vaccine ingredients intact during storage. Also, stabilizers keep vaccine ingredients from sticking to the side of the vaccine vial. Examples of stabilizers include sugars (lactose and sucrose) and amino acids (glycine and monosodium glutamate) [118].

8.4. Preservatives

Preservatives are used in some vaccine formulations to eliminate fungal and/or bacterial infections in vaccines. Preservatives were initially introduced to avoid bacterial contamination in multi-dose vials (not used in individual vial vaccines). Thiomersal, phenoxyethanol, and phenol are among the preservatives utilized. Preservatives have been used in numerous vaccinations, although there have been relatively few significant harmful effects linked with their use worldwide, such as allergy [119].

8.5. Trace components

Trace components in vaccines are the minute amounts of chemicals or substances used in the early stages of the manufacturing or leachables, e.g., from container. Depending on the production procedure, trace quantities of cell culture fluids, egg proteins, antibiotics, or inactivating agents can be present [119]. In certain instances, only small amounts of these compounds are found in the final vaccine component.

Antibiotics are occasionally utilized during the processing phase to guarantee that contamination of bacteria does not arise. Some influenza vaccines contain antibiotics such as Neomycin, Polymyxin B, and/or Gentamicin. Moreover, prefilled vaccines syringes may contain traces of silicon oil that uses as a lubricant in prefilled vaccines syringes.

During the development of WIV and split-virus inactivated vaccines, inactivating agents are used, and the final vaccine contains relatively few of these inactivating chemicals or splitting agents, such as formaldehyde, glutaraldehyde, or surfactants, respectively [70].

9. Novel Influenza Vaccine Platforms

9.1. Virus-like Particle (VLP) Vaccines

VLPs are composed of surface viral glycoproteins that self-assemble to form non-replicating particles that look like viruses. These particles mimic the native viral structure but lack genetic materials and cannot cause an infection [120]. VLPs were successfully developed as vaccines and have been used against several viruses, including hepatitis B virus (Heptavax-B, Engerix-B®, H-B-Vax®II, and Hepavax-Gene®), human papillomavirus (Gardasil®, Cervarix®, Cecolin®, and Gardasil-9®), and hepatitis E virus HEV (Hecolin®) [121]. Therefore, VLP platform has proven to be a good strategy to produce vaccines including influenza. For example, VLPs have been combined with hepatitis B virus core (HBc), and a

recombinant M2 protein containing three tandem copies of M2e (3M2e), as well as nucleoprotein (NP) epitopes to overcome the challenge of rapid influenza evolution [122]. Innate immunity can be activated by VLPs directly or by stimulating antigen-presenting macrophages or dendritic cells, which can lead to efficient induction of virus-specific T- and B-cell responses. Moreover, influenza VLP vaccines can contain several different viral proteins (HA, NA, M1, or M2e) in combination or individually on single VLPs, providing some flexibility in vaccine design for broader protection. The addition of pure viral neuraminidase to a traditional influenza vaccination resulted in a balanced and wide immune response. Moreover, because of the protein's conserved nature, M2e is a potential target for universal vaccine development [123]. Some of the VLP influenza vaccine candidates have reached clinical trials (Table 2). A recombinant A (H1N1) 2009 influenza VLP vaccine (HA) by Novavax was evaluated in phase 2 clinical trials in 2012 for safety and immunogenicity [124]. This VLP influenza vaccine showed a robust immune response after a single vaccination, with only mild adverse events. Moreover, a plant based QVLP (HA) by Medicago has completed the phase 2 clinical trials in 2019 [125]. The plant-based VLP influenza vaccine was able to induce robust antibody responses and antigen-specific CD4+ T cells. These VLP vaccine candidates were able to produce humoral and/or cellular responses. Also, it showed cross-protection by inducing antibodies against four different influenza strains (two influenza A and two influenza B) [125].

9.2. Antigen-Presenting Cell (APC) Inducible Vaccines

CD11c+ dendritic cells (DCs) contribute significantly to adaptive antiviral immune responses during viral infection by accumulating and converting viral antigens into peptides

for presentation to T-cells in secondary lymphoid organs through the main histocompatibility complex (MHC) [133].

Some pre-clinical work has been done to strengthen the T-cell response to the influenza virus by inducing the antigen-presenting cells. For instance, Fonteneau and colleagues have studied the in vitro impact of influenza virus infection on blood-purified DCs and their capacity to accumulate and present viral antigens to CD8+ and CD4+ T-cells. They demonstrated that exposure to influenza virus induces an expansion of anti-influenza virus T helper 1 (TH1) CD4+ T-cells and cytotoxic T lymphocytes CTLs. These findings indicate to a novel function for DCs in the generation of antiviral T-cell responses, suggesting that these DCs play an important part in the adaptive immune response to viruses [134]. Moreover, another approach has shown that grafting the alpha-Gal epitope onto HA enhances APCs' influenza vaccine virus uptake [135]. Thus, immunogenicity of influenza virus vaccines may be enhanced by efficiently targeting vaccinations to APCs. Additionally, influenza HA can be targeted to APCs using fusion DNA vaccines which encode proteins that bind to influenza HA bivalently to target them to distinct surface molecules on APCs. This targeting was able to induce the immune response toward either CD8+/Th1 T cell response or antibody/Th2 response [136].

Table 2. Current VLP influenza vaccine candidates in preclinical and clinical trials.

VLP vaccine candidate	Type of response	Cross-protection	Clinical phase	Type of species	Sponsor	References
BV VLP-HA-NA-M1	Humoral and cellular	N/A	Pre-clinical	Mice	-	[126]
HBc VLP-M2e-HA2 (Tandiflu1)	Cellular	Yes	Pre-clinical	Mice	-	[127]
HBc VLP-M2e-NP	Humoral and cellular	Yes	Pre-clinical	Mice	-	[122]
Influenza VLP-HA (H1, H8, H13, H3, H4, H10)	Humoral	Yes	Pre-clinical	Mice	-	[128]
Recombinant A (H1N1) 2009 influenza VLP vaccine (HA)	Humoral	N/A	Phase II	Human	Novavax	[129]
HA and M2e5x	Humoral and cellular	N/A	Pre-clinical	Mice	-	[130]
Plant-based QVLP (HA)	Humoral and cellular	Yes	Phase I &II	Human	Medicago	[131, 132]

9.3. Nanoparticle-based Influenza Vaccines

Conventional influenza vaccines can provide systemic protection against the virus; however, local protection in the mucosal respiratory is also desirable against the influenza virus

[137, 138]. Nanoparticles have characteristics such as solubility and stability that make them suitable for mucosal immunity as they induce immunity that works against respiratory viruses. These nanoparticles can be derived from a natural or synthetic source and work as antigen carriers [139]. Thus, more studies have been done using nanoparticles against the influenza virus than other viruses. Papaya mosaic virus nanoparticle was used as an adjuvant with trivalent inactivated flu vaccines, and it has been tested to immunize mice. This nanoparticle vaccine was found to be superior to IIV in terms of induction of anti-influenza immunity based on IgG2, IgA, and IgG titers in bronchoalveolar lavage samples and serum, especially after intranasal immunization [140].

Moreover, a polylactic-co-glycolic acid (PLGA) nanoparticle was conjugated to influenza A (H1N1) conserved peptides and was intranasally administered as a vaccine. This vaccine was able to induce antigen-specific CD4+ and CD8+ T-cells and consequently induce protection in the lungs of pigs [141]. Therefore, the induction of cross-protective antibodies that can target several pathways involved in the infection would be highly beneficial for better protection against the influenza virus. Two different conserved influenza antigens (helix C and the ectodomain of matrix protein 2 (M2e)) were linked in a self-assembled nanoparticle to achieve this goal. They were able to induce neutralizing antibodies in mouse models [142].

9.4. Universal Influenza Vaccines

Although vaccination is the most efficient way to prevent influenza infection, antigenic shift and antigenic drift of influenza viruses allow them to escape antibody neutralization [143]. These variations in the influenza viruses are unpredictable, so influenza outbreak management is very challenging. To overcome the limitations of the current influenza vaccines, a universal influenza vaccine that provides long duration and broad protection would be highly desirable.

An ideal universal influenza vaccine should be effective against all influenza viruses (A and B) HA and NA subtypes or antigenic mutations. To reach this goal, the vaccine should induce cross-protective antibodies, which might be achieved by targeting the conserved epitopes found in HA, NA, or M2 or internal proteins such as M1 and NP.

The stem or stalk region of HA is highly conserved across different strains of influenza viruses and is considered a very promising target for developing a universal influenza vaccine [144]. Furthermore, some of the isolated antibodies from humans generated against the stalk region of the virus neutralize all influenza A virus subtypes, which can be used for developing a universal influenza vaccine [145]. For example, a sequential chimeric HA vaccine technique was developed to redirect the immune response from the head to the stalk domain (Table 3). Chimeric HAs are composed of stalk domains from group 1 or group 2 influenza viruses and head domains from subtypes of avian influenza virus. After completing a phase I clinical trial in 2020, this universal influenza vaccine candidate was found to be safe and evoke a broad, vigorous, long-lasting, and effective immune response directed against the conserved, immunosubdominant stalk of the hemagglutinin [146].

Table 3. Universal influenza vaccine candidates in preclinical and clinical trials.

Vaccine candidate	Vaccine type	Manufacturer	Clinical phase	Participants	Mechanism of action
Chimeric HA proteins [146]	Hemagglutinin-based	Glaxo-SmithKline	Phase I	66	Ag-specific cellular response Broadly cross-reactive Abs

Computationally optimized broadly reactive antigens (COBRA) [148]	Computationally optimized antigens	Sanofi-Pasteur	Pre-clinical	-	Elicitation a unique broad cross-reactive and cross-neutralizing Ab against HA.
NP, M1 and HA peptides (Multimeric-001) [149]	Recombinant proteins	BiondVax Pharmaceuticals Ltd/NIAID	Phase III	12463	Cellular (B- and T-cell) immune response.

Moreover, a computationally optimized broadly reactive Ags (COBRA) targeting H1 was developed as a universal influenza vaccine (Table 3). To evaluate the breadth of B cells, antibody-secreting cells elicited by a COBRA hemagglutinin (HA) (termed P1) were compared to antibody-secreting cells elicited by historical H1N1 vaccine strains. P1 HA-elicited monoclonal antibodies (mAbs) demonstrated an extensive range of HA identification, spanning from narrowly to widely reactive mAbs. Also, a new vaccine, Multimeric-001, was developed to defend against seasonal and pandemic influenza virus types, independent of mutations, by containing conserved linear epitopes from the HA, NP, and M1 proteins of influenza type A and type B strains (Table 3). This vaccine was evaluated in phase III clinical trial in 2020 to assess its protection and tolerability, as well as the humoral and cellular immune responses [147]. The vaccine was well-tolerated, and no serious adverse effects were recorded. The humoral and cellular responses indicate that the vaccine has cross-immunity against influenza virus strains independent of mutations. Participants given Multimeric-001 dose prior to a seasonal vaccination had greater antibody response to match the H1N1 and H3N2 strains compared to seasonal vaccination alone. Moreover, an increase in CD4+ and CD8+ T cell

responses to H1N1, H3N2, and influenza B relative to baseline in those who were vaccinated with Multimeric -001.

10. Characterization of Vaccine Products

Most of the vaccine production time (70%) is devoted to quality testing [150] to assure purity, potency, and vaccine safety. During the development of influenza vaccines, extensive characterizations are required to ensure that the manufacturing steps follow the current good manufacturing practice (cGMP). Although the production of vaccines differs, quality control tests must consider certain aspects of physicochemical parameters such as purity, quantity, homogeneity, and stability.

10.1. Purity

The purity of the vaccine is a critical physicochemical criterion for determining the proportion of 'active' vaccine antigens in the final vaccine product. Ultimately, the degree of pureness measured by the vaccine antigen will rely on the test's specificity, additional methods for quantification and preparation of the sample of the product, and non-product impurities. Thus, it is critical to employ selective assays to measure putative non-product/process-related impurities (e.g., host cell proteins, host cell DNA and/or RNA, lipids, cell culture inducers, etc.) and product-related impurities (e.g., molecular weight variants such as aggregates and degradants) to delineate the existence of contaminants and impurities below a defined amount [151]. Moreover, IIV need to be purified by removing the inactivating agent using filtration, centrifugation, or evaporation methods. Splitting materials in split-virus vaccines such as Triton-100 need to be removed in the final product, which can be done by filtration or other separation methods such as hydrophobic interaction chromatography [152]. Table 4 summarizes several approaches that may be used, often in combination, to determine vaccine purity. For example, using orthogonal processes such as mass spectrometry, the molecular

mass can be accurately measured, and the covalent change (i. e. glycosylation) identity and locations may also be detailed. Furthermore, since an impurity can exhibit the same mass/charge (m/z) ratio as the vaccine antigen during mass spectrometric analysis, using another orthogonal method such as light scattering (size-exclusion chromatography, multiple angle laser light scattering) that measures mass based on the radius of gyration helps in separating the vaccine antigen from the unresolved impurity during mass spectrometric analysis.

10.2. Quantity

HA is the primary antigenic protein in influenza virus, and the quantity of HA should be enough in the vaccine to induce the immune system. Thus, HA quantity must be 15 µg/ml for any type of influenza vaccines [152]. Quantification is carried out using conventional protein estimation approaches such as UV Abs 280nm, bicinchoninic acid assay, Bradford assay, Lowry assay, detergent-based fluorescent detection, quantitative gel electrophoresis with fluorescent staining, amine-labeling 'derivatization' (using fluorescent probes), and ELISA (enzyme linked immunosorbent assay). Although each approach has some possible advantages and disadvantages, it is critical to understand the method(s) of measuring in relation to the sample volume, pH environments, sensitivity, throughput, robustness, and precision of the assay [153]. Finally, assessing the quantity of the 'working' vaccine antigen in the presence of product-related impurities is critical for developing correct dose guidelines.

10.3. Homogeneity

Given that the size or molecular weight of the protein/glycoprotein in the vaccine antigen can be determined using many techniques, separation and scattering methods are the main two methods [154]. In the separation method, a force can be applied (e.g., mechanical, centrifugal,

electrical) on the sample, then the size of the biomolecule can be determined depending on its movement. In contrast, the size of the biomolecule in the 'scattering process' is measured by the association of incident radiation (e.g., light, x-rays) with the biomolecule. Then, there is a transition to the "transport system" as the most often employed method of determining size of proteins including gel-filtration (size-exclusion) chromatography, native/SDS-gel electrophoresis, sedimentation equilibrium, mass spectrometry, sedimentation rate, and field-flow fractionation (Table 4). Field-flow fractionation is a technique that separates proteins/glycoproteins/particles depending on the orientation of the laminar flow of the molecules in a narrow channel [155], with smaller molecules flowing faster and larger molecules (that cannot disperse rapidly) moving slowly. Also, the usage of mass spectrometry to assess the molecular mass or scale of proteins aids in protein recognition and quantifies protein purity, covalent alteration, and degradation products at both quantitative and qualitative stages [156]. Light scattering and electron microscopy (EM) are the two often used for characterizing and determining the size of protein/glycoprotein-based vaccine antigens or the size distribution of virions in WIVs.

Moreover, the presence of protein aggregation in the vaccine product can affect the stability and efficacy of the vaccine [157]. These aggregates can be caused by removing the splitting agent (surfactant) in the manufacturing of split-virus influenza vaccines [158]. In addition to EM and light scattering, UV-Vis absorption spectroscopy and fluorescence emission spectroscopy are reliable techniques in identifying protein aggregations [71, 157]. EM is a direct imaging tool for determining the size and shape of large proteins or the complex of proteins. However, owing to the high expense of instrumentation and the specialized knowledge needed for such experiments, other techniques (such as light scattering) are often

chosen as primary methods for size analysis. Molecular weight, distribution, diffusion coefficients and hydrodynamic radius can be measured through dynamic light scattering.

10.4. Stability

Understanding the function and stability of the protein/glycoprotein vaccine antigen is crucial to the vaccine's general effectiveness in eliciting the required protective immune response. The instability of vaccines may be induced by different factors such as heat, light, radiation, environmental changes, or interactions with the container or other vaccine ingredients. Excessive heat or freezing might hasten the loss of vaccine's potency. It is thus necessary to treat vaccines with care, pay close attention to their handling to guarantee that they are of high quality, and provide maximum efficacy. This may be accomplished via the use of cold chains to keep vaccines in a restricted temperature environment (which is required for potency preservation) from the producer all the way down to the end consumers. Moreover, in split-virus vaccines, surfactant type, incubation time, virus concentration, surfactant concentration, and virus split ratio are other factors that could affect vaccines stability. For example, aggregation may present after diluting or removal of the surfactant, so the remaining surfactant concentration should be monitored by optimizing the number of filtering steps to maintain vaccine stability and efficacy [158].

A combination of analytical methods can be used to study vaccine stability, such as fluorescence emission spectroscopy, UV-Vis absorption spectroscopy, and Hemagglutinin inhibition assay (HI) test (Table 4). Because aggregation and/or degradation are typical characteristics that influence stability for proteins/glycoproteins, utilizing these analysis methods helps identify the size or molecular weight of the aggregation to understand vaccines' short-term and long-term stability [159].

Table 4. Techniques in vaccine development and characterization.

Assay or Technique	Use	References
Identity		
Hemagglutinin inhibition assay (HI Test)	Selection of the candidate virus vaccine CVV and stability	[160-162]
Single radial immunodiffusion assay SRID	Vaccine potency	[163-165]
Enzyme-linked immunosorbent assay ELISA		
Sedimentation		
Velocity	Mass and Size	[166]
Equilibrium	Mass, association	[166]
Chromatography		
Hydrophobic interaction	Purification	[167]
Reverse phase	Purification and stability	[168]
Ion exchnageexchange	Charge	[169]
Size exclusion	Size	[170]
Affinity	Specific interaction	[171]
Light scattering		
Dynamic Light Scattering DLS	Size measurements and aggregation study	[172, 173]
Static light scattering		

UV-Visible absorbance spectroscopy	Agglomeration assessment and stability	[157, 173, 174]
Mass spectrometry	Protein quantification, Vaccine potency	[175, 176]
Fluorescence spectroscopy	Protein stability, Agglomeration assessment	[71, 157]
Microscopy		
Transmission electron microscopy TEM	Structure-guided information and aggregation study	[71, 157, 173]
Flow imaging microscopy	Size	[177]

11. Effectiveness of influenza vaccines

Throughout each flu season, studies are conducted to assess the effectiveness of seasonal flu vaccination in order to evaluate how effectively flu vaccinations are performing. The efficacy of vaccines has been shown in studies to differ depending on the population investigated, the research design, the outcome (s) assessed, and the flu vaccination season analyzed. According to the CDC, flu vaccination lowers the risk of influenza disease by between only 40% and 60% in the general population during flu seasons when the majority of circulating influenza viruses are closely matched to those used in flu vaccines. Moreover, a poorly matched flu vaccine will offer little or no protection against disease caused by the viruses it doesn't cover, but it will still provide immunity against the other influenza viruses prevalent that season (Fig. 2-6).

While much emphasis is paid to the viral factors that contribute to inconsistency in vaccination responses, other factors can affect the results of influenza vaccine efficacy such as

host factors and study design factors. A growing body of data suggests that host variables such as age, biological sex, pregnancy, and immunological history all play significant roles in modifying the effectiveness of influenza virus vaccines. Thus, A better understanding of the host variables that influence influenza vaccine-induced immunity may help enhance existing seasonal and future universal influenza vaccines by optimizing or even customizing vaccine type, dosage, and adjuvant usage. To study the influenza vaccine's effectiveness, randomized controlled trials (RCTs) are the most trustworthy because they are less vulnerable to selection and confounding biases. However, the test-negative design (TND) has been widely used for measuring influenza vaccination efficacy since 2004. Although it minimizes selection bias associated with health seeking activities, it has limitations in only including people who seek care for respiratory illnesses.

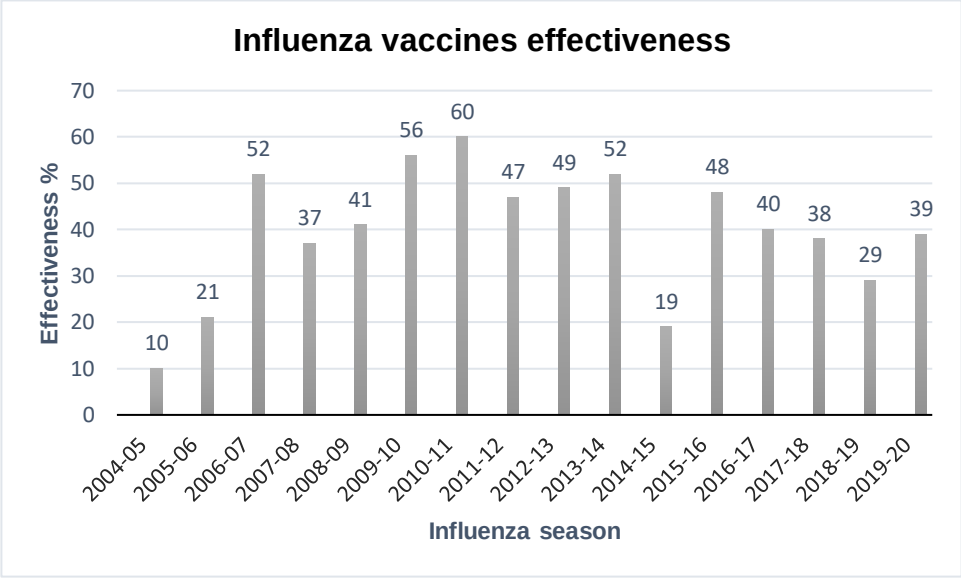

Figure 2-6: Seasonal influenza vaccines effectiveness in US, according to CDC.

12. Conclusions

Due to extensive viral shift and drift, influenza can evade the host immune defense, and hence, remains to be one of the significant recurring global public healthcare challenges. Therefore, there is a need to update the influenza vaccines annually. Currently, three types of

influenza vaccines are on the market: inactivated influenza vaccine (IIV), live attenuated influenza vaccine (LAIV), and recombinant hemagglutinin (HA) vaccine. Like most other vaccines, the influenza vaccine has a complex formulation that requires several key components such as a viral antigen, adjuvant, preservative, and stabilizer to maintain a stable and effective vaccine formulation. Further, the vaccine undergoes many quality tests throughout the processing steps and the final product to assure that the vaccine is up to standard and safe to use. Nevertheless, more research is needed to overcome the limitations of current influenza vaccines, such as low efficacy, long production time, and the lack of cross-protection. To this end, new influenza vaccine candidates are in progress to improve the vaccine efficacy or to provide cross-protection against many different strains (i.e., a universal influenza vaccine) to overcome the need for an annual update for the vaccine.

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CHAPTER 3

Triton X-100 alternative surfactant for split-virus influenza vaccine

CHAPTER 3- Chapter Overview

The following chapter is a full research article.

The European Commission has designated Triton X-100, a splitting agent utilized in the production of split-virus influenza vaccines, as a "substance of very high concern" owing to the production of environmentally hazardous metabolites that occur following its release into the environment. In Chapter 3, an experimental study has been presented, showcasing a potential alternative to Triton X-100 that exhibits promising characteristics for use in the manufacturing of inactivated influenza vaccines.

Abstract

The best way to protect against the spread of influenza is via vaccination. The most widely used influenza vaccinations are inactivated split-virus vaccines due to their proven safety and the inexpensive cost of their manufacture. Chemical alteration with substances like formaldehyde or beta-propiolactone, as well as physical manipulation using ultraviolet (UV) radiation or gamma irradiation, are all viable options for inactivating influenza viruses. Inactivated flu vaccines are often prepared by splitting the virus using a non-ionic surfactant like Triton X-100. The European Commission has labelled Triton X-100 a "substance of very high concern," therefore it may only be used in extremely specific situations as of January 2021.

Tween-20, Tween-80, and Brij-56 were among the surfactants we tested as possible replacements for Triton X-100 in the production of inactivated vaccines. Many different types of spectroscopy and microscopy were used to analyse surfactant-virus interactions and determine how effectively they might break up the virus: fluorescence, light scattering (NanoSight and dynamic light scattering), and transmission electron microscopy. Our results show that Brij-56 has a concentration range in which it is just as effective as Triton X-100 in splitting the influenza virus. Similar findings were reported when we tested the effects of surfactants on other influenza strains, including A/Switzerland/9715293/2013 (H3N2) and B/Phuket/3073/2013 (H3N2).

The surfactant Brij-56 has several uses and is thus commonly used in both commercial settings and academic labs. In manufacturing, it is used as a surfactant and emulsifier. Brij-56 has several uses in scientific labs, including those related to cell culture, protein extraction, and molecular biology. Brij-56 is preferable to other compounds like Triton X-100 since it doesn't do any harm to the environment. Its widespread use demonstrates its adaptability and

positive qualities for a wide range of scientific and industrial applications, including the production of vaccines.

Overall, our findings support the idea that Brij-56 might replace Triton X-100 in the manufacturing of inactivated influenza vaccines, thereby providing a safer and more environmentally friendly option.

1.Introduction

The influenza virus is known to have significant social and economic impacts, resulting in an annual expenditure of 3 to 5 million cases of severe illnesses and between 290,000 to 650,000 respiratory-related deaths globally [2]. Nevertheless, there have been reports indicating a significant decrease in influenza activity, implying that the strategies implemented to combat COVID-19 were successful in mitigating the transmission of other respiratory viruses, such as Influenza [3, 4]. it is anticipated that the activity of these viruses, including Influenza, may rebound and result in a resurgence of cases as certain preventive measures are lifted or relaxed. Therefore, the World Health Organization (WHO) recommends that in future influenza epidemics, certain community mitigation measures could be applied to minimize transmission, especially among populations most vulnerable to severe illness or complications [5].

This underscores the critical importance of producing high-quality vaccines in a timely manner. At present, vaccine development largely relies on trial-and-error methods. Furthermore, the limited window available each year for the preparation of seasonal influenza vaccines makes this task particularly challenging, further emphasizing the need for efficient and effective vaccine production processes.

Surfactants have become a common component of most influenza vaccines available on the market, with the primary purpose of breaking up the full, intact virus [2]. Subsequently, these surfactants are subjected to removal processes; however, traces of the surfactants may remain. Triton X-100, in particular, has demonstrated moderate, non-denaturing surfactant properties, effectively solubilising surface-exposed membrane glycoproteins such as hemagglutinin (HA) and neuraminidase (NA), as well as other viral proteins[6]. The surfactant plays a crucial role in the generation of split vaccines by disrupting the lipid membrane of the viruses, resulting in partial inactivation. It not only acts as a viral disintegrator, but also as a vaccine stabiliser and

homogenizer, keeping biomolecules from clumping together and dropping out of solution [1].The efficacy of split viral vaccines relies heavily on the immunogenicity of solubilised particles and preservation of 3D protein structures [7]. Due to its ability to solubilise viral proteins while preserving their structures, Triton X-100 facilitates the development of highly immunogenic split virus vaccines with significantly reduced reactogenicity as compared to whole virus vaccines.

Recent studies have revealed that Triton X-100, upon discharge into the environment, generates ecologically harmful metabolites. Of particular concern is the phenyl component (4,(1,1,3,3) tetramethylbutylphenol), which exhibits resistance to biodegradation and may disrupt the reproductive cycles of aquatic organisms [8]. Consequently, the European Chemicals Agency has imposed usage limits on this substance [9]. This has led to challenges for major Triton X-100 customers, such as the pharmaceutical sector, in their search for a replacement surfactant that meets environmental sustainability standards while also being effective in splitting virus [10].

An ideal replacement candidate would be capable of fulfilling Triton X-100's primary function while exhibiting properties similar to Triton X-100 during production. Specifically, it should not adversely impact delicate biomolecules of interest, be soluble in water with low foam propensity, have straightforward and easily validated analytics, and be easily removed via purification steps. Furthermore, it should have a desirable biodegradability and pharmacological toxicity profile. If such a candidate detergent is identified, the switch from Triton X-100 could be achieved with minor modifications to the production process, and regulatory submissions may only require the Chemistry, Manufacturing, and Controls Section rather than more rigorous preclinical or clinical evaluations.

In the context of planning, it is advantageous to identify a prospective replacement for Triton X-100 that exhibits minimal differences in properties. In this regard, a nonionic polyethylene glycol (PEG) surfactant based would be an ideal candidate. PEG is known for its biocompatibility and biodegradability, making it a suitable option for applications involving biological systems. Moreover, the presence of a low critical micelle concentration (CMC) is highly advantageous in a potential substitute surfactant. A low CMC signifies the surfactant's ability to efficiently form micelles even at lower concentrations. This quality is particularly useful in situations when just a little quantity of surfactant is desired, such as in vaccine manufacture.

Many alternatives to Triton X-100 as a surfactant for inactivating certain viruses have been presented recently [8, 11, 12]. Nevertheless, they have not been thoroughly examined for their potential to inhibit the spread of the flu virus. To fill this informational need, we studied the viability of using alternative surfactants to Triton X-100 in the production of inactivated influenza vaccines, namely Tween-20, Tween-80, and Brij-56 (Fig.3-1). All of the investigated surfactants are commercially available and have several biochemical uses, such as in emulsifying, cell lysis, protein solubilizing, and nanostructure creation. Polyethylene glycol hexadecyl ether (Brij 56) has antifungal effects as well [13]. It is important to note that, like Triton X-100, these surfactants may cause hemolysis at high concentrations; however, this is not an issue in vaccine manufacture since all surfactants must be removed from vaccine formulations after inactivation. Because of its low CMC, Brij 56 has a significant advantage over competing options, allowing for its usage at manageable quantities.

We used many methods, such as transmission electron microscopy (TEM), fluorescence microscopy, light scattering (NanoSight, dynamic light scattering), and spectroscopy. We also examined the impact of these surfactants on various influenza strains: A/California/7/2009 (H1N1), A/Switzerland/9715293/2013 (H3N2), and B/Phuket/3073/2013 (H3N2).

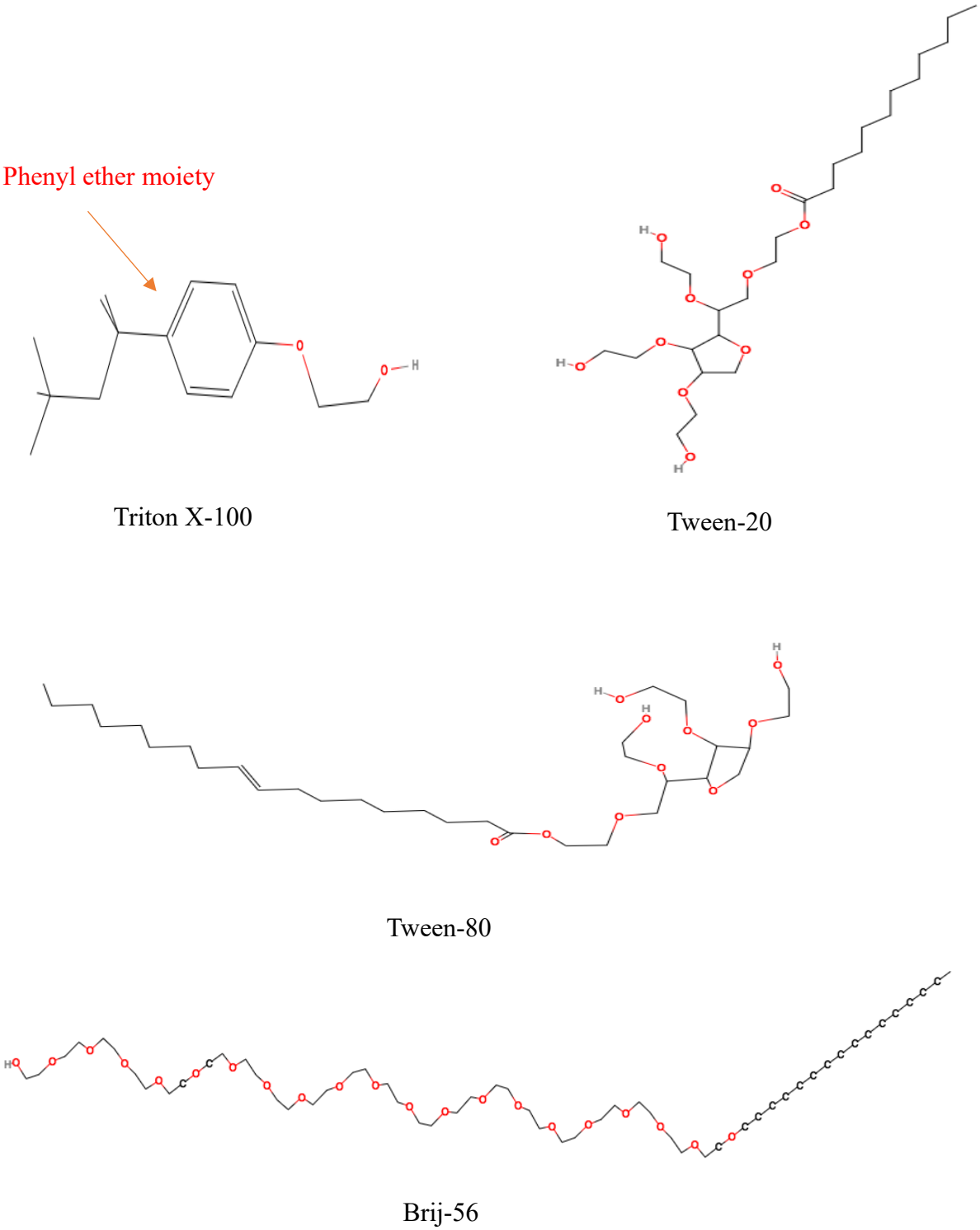

Fig. 3-1. Different surfactants as alternatives for Triton X-100 including Tween-20, Tween-80, and Brig-56. All of the alternative surfactants lack the phenyl ether moiety.

2.Materials and methods

2.1. Materials

All influenza virus samples were donated by Sanofi-Pasteur (Pennsylvania, USA). The strains studied were B/Phuket/3073/2013 (H3N2), A/California/7/2009 (H1N1), and A/Switzerland/9715293/2013 (H3N2). Triton X-100, Brij-56, Tween-80, and Tween-20, Nile red (NR), and methanol were purchased from Sigma-Aldrich (Castle Hill, AUS). Copper TEM grids coated with carbon and Formvar were acquired from ProSciTech (Queensland, AUS). Deionized water was used to dilute the samples. To prevent aggregation and/or dissociation of produced aggregates, mixing was done by gentle pipetting. A Hellma quartz microcuvette (New York, USA) was utilized for all spectroscopic studies. GraphPad Prism 9 was used to analyze the data.

2.2 Fluorescence emission spectroscopy

The fluorescence emission spectrum of Nile red was recorded from 500 to 800 nm, with excitation at 490 nm, using a Horiba Jobin Yvon Fluorolog FL-322 (New Jersey, USA) with 4 nm slits and 0.15 second integration time. Each sample was evaluated using at least two separate aliquots (n = 2 or more), with necessary correction made for each spectrum by subtracting out the background signal. A 2 mM stock solution of Nile red was prepared in methanol and stored at 8°C. Then, 25 μL of this stock solution was added to 1000 μL water to prepare another diluted stock solution with 50 μM concentration. For each sample, 30 μL of the 50 μM Nile red stock solution was added to 70 μL of the sample (100 μL total). To observe time-dependent viral splitting, measurements were taken at 3, 5, 10, 20, and 50 minutes.

2.3 Dynamic light scattering (DLS)

The viral splitting process was monitored by analysing the aggregation and monomer size distributions of influenza virus samples before and after adding different surfactants using the Zetasizer Nano ZS (Malvern, United Kingdom). The samples were treated with 0.1% w/w Triton X-100 or Brij-56 for 20 minutes, and then diluted to 1 mL with distilled water before being pipetted into 1 mL disposable plastic cuvettes and measured at 25°C. The refractive index of influenza virus in water was set to 1.45, with an absorption value of 0.001.

2.4 Transition electron microscope (TEM)

TEM images of influenza viruses were obtained using a JEOL JEM-1400 instrument (Tokyo, JPN). Three whole influenza virus samples were prepared for imaging, with one sample serving as a control, and the remaining two samples were treated with 0.1% Triton X-100 or Brij-56 and incubated for 20 minutes before being loaded onto TEM grids. Negative staining was performed using a 2% w/v ammonium molybdate solution in water for one minute. The grids were then blot-dried on filter paper and allowed to air-dry before imaging.

2.5 Nanoparticle tracking analysis (NTA)

The virus samples were diluted with water to a final volume of 1 mL. The camera was adjusted to ensure that all particles were clearly visible, and the parameters were set according to the manufacturer's instructions (NanoSight NS300 User Manual, MAN0541-01-EN-00, 2017). The autofocus system was adjusted to exclude any indistinct particles. Five 1-minute videos were recorded for each measurement, with the following settings: cell temperature of 25°C and syringe speed of 40 μL/s. The captured videos were analyzed using the built-in NanoSight Software NTA 3.1 Build 3.1.46 with a detection threshold of 5.

3. Results and discussion

3.1 Fluorescence emission spectroscopy

Nile red (NR), a hydrophobic fluorescent dye, is capable of binding to a variety of hydrophobic substances found in biological samples, such as lipids [14-20], proteins aggregates [21-24], surfactant micelles [25, 26], the hydrophobic regions of certain proteins [14, 22]. As a result, NR fluorescence emission has numerous biological applications. Alterations in polarity and hydrophobicity of the microenvironment can modify the intensity and wavelength of the fluorescence signal [15, 19, 27].

In aqueous environments, the quantum yield of NR is observed to be relatively low as shown in Fig.3-1. However, the fluorescence intensity increased considerably upon the addition of surfactants, indicating that NR molecules were encapsulated within micelles during their formation, consistent with prior research findings [27, 28].

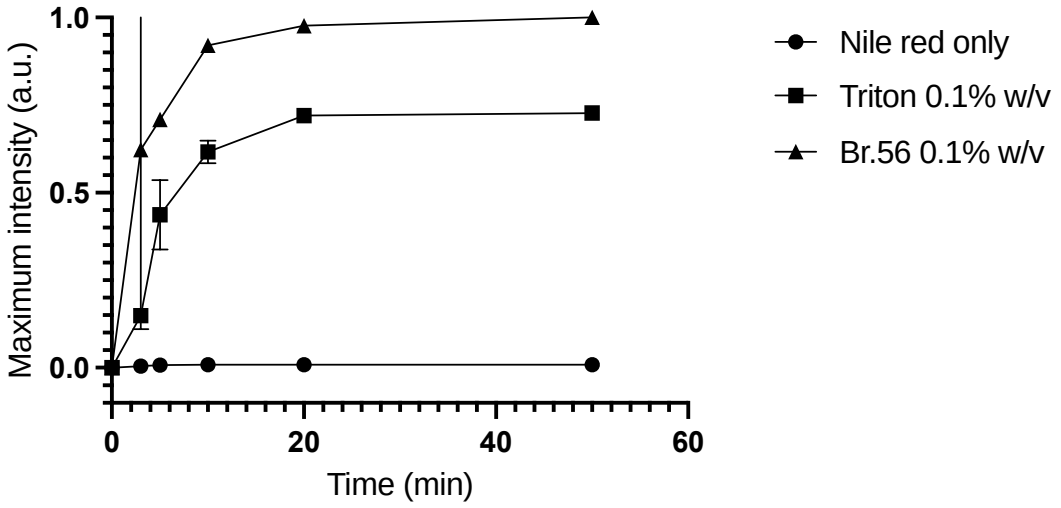

Fig. 3-2. The change of fluorescence intensity as a function of time for Nile red only in water, Nile red with 0.1% w/v Triton X-100 or Brij-56 only without virus.

The presence of viruses and other hydrophobic particles, such as protein aggregates, provides more hydrophobic environments for NR to bind to, which results in increased fluorescence. In this study, after adding the surfactants alone, NR intensity increases for all surfactants at their respective concentrations until it stabilizes at around 20 minutes (Fig.3-2), which is consistent with previous findings on the time required for NR intensity to stabilise [29, 30]. Also, after adding the virus with the surfactants, the change in NR intensity was similar to that observed when introducing the surfactants alone, suggesting that the NR intensity change is primarily related to the creation of micelles (Fig.3-3). The increased NR fluorescence intensity may be attributed to the lipid membrane of the virus and hydrophobic components of the virus that have been split. Despite the lower critical micelle concentration (CMC) of Tween-20 (0.007%w/v) compared to that of Triton X-100 (0.02%w/v), the NR intensity is greater with Triton X-100. Triton X-100 may exhibit better solubilization properties for hydrophobic components, including the ones that interact with NR. It could effectively disperse and solubilize hydrophobic substances, allowing NR to interact more efficiently with the targeted molecules, resulting in higher fluorescence intensity. Brij-56 had the highest NR intensity of all the surfactants tested at different concentrations, likely due to the combination of its low CMC (0.004%w/v) that lead to form a higher number of micelles at the experimental concentrations used. This higher micelle formation can provide a greater hydrophobic environment for NR to bind and enhance its fluorescence intensity. Moreover, Brij-56 might have specific interactions or binding affinity with the hydrophobic components or structures of interest that lead to enhanced NR intensity. These interactions can influence the microenvironment surrounding NR, resulting in increased fluorescence emission. This was observed at concentrations of 0.05% and 0.02%, and there was no significant difference between the various surfactants at these concentrations, despite their different CMCs. At the lowest concentration (0.005%), which is lower than the CMC of Triton X-100 (0.02%), there

was no increase in NR intensity with time due to the absence of micelles formation. In summary, our findings suggest that Brij-56 may be an effective substitute for Triton X-100.

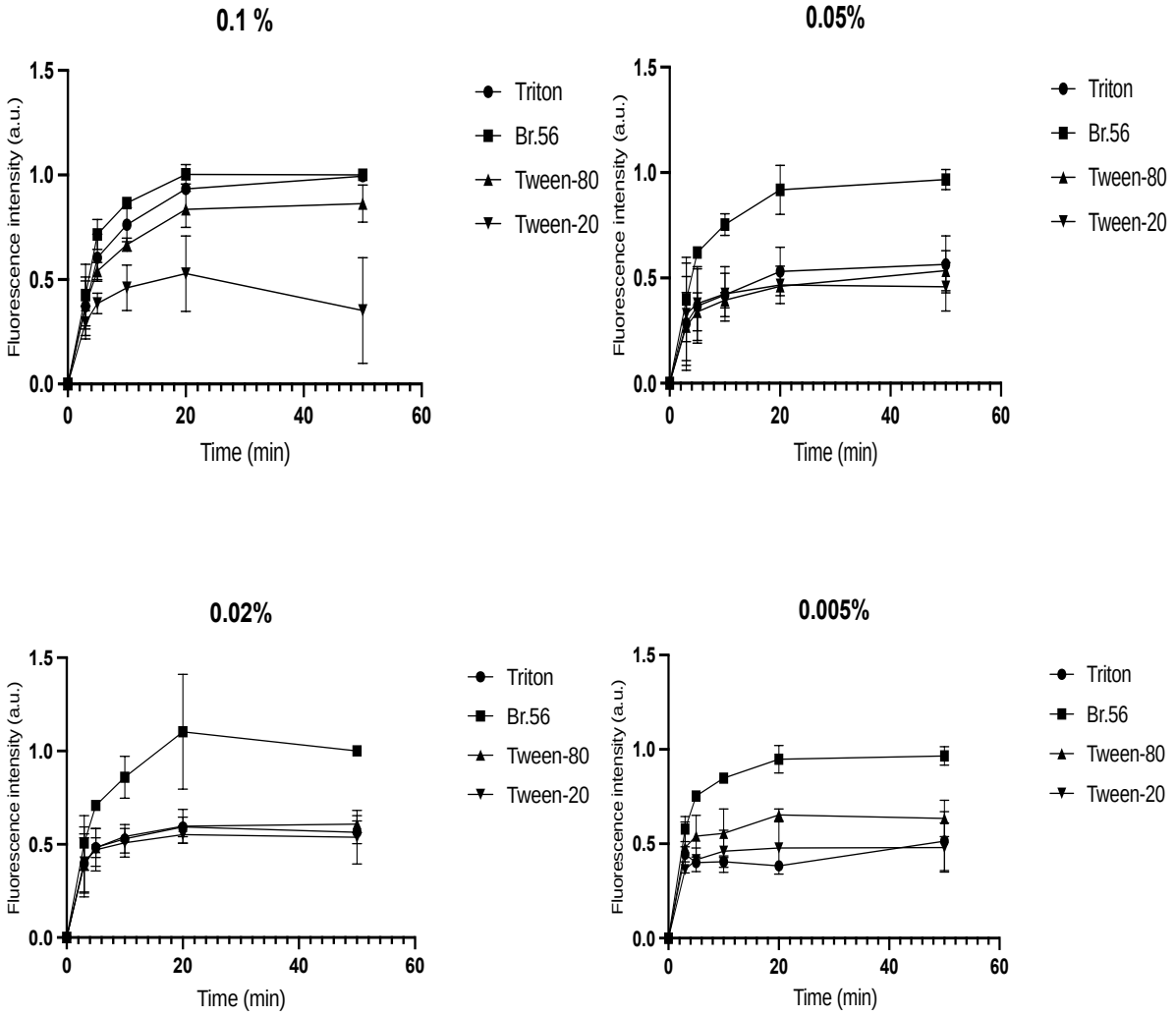

Fig. 3-3. The change of fluorescence intensity as a function of time after incubation of influenza virus (A/California/7/2009 (H1N1)) with surfactants at different concentrations.

Also, we conducted experiments on A/Switzerland/9715293/2013 (H3N2) and B/Phuket/3073/2013 (H3N2) influenza virus strains to assess Brij-56's efficacy as a splitting agent in comparison to Triton X-100 using two different concentrations (0.02 and 0.005% w/v) (Fig. 3-4). The results showed that both surfactants increased the intensity of NR at their respective concentrations until it stabilized after approximately 20 minutes. At a concentration of 0.05% with A/Switzerland/9715293/2013 (H3N2), there was no significant difference between Triton X-100 and Brij-56 during the first incubation times. However, at 20 and 50

minutes, Brij-56 demonstrated increased NR intensity, possibly due to the increased number of Brij-56 micelles and its splitting activity, which provides a greater hydrophobic portion for NR to bind to as compared to Triton X-100. Similar to our previous findings with A/California/7/2009 (H1N1), Triton X-100 had no effect on the NR intensity with B/Phuket/3073/2013 (H3N2) at varied incubation times at 0.005%, which is below its CMC. On the other hand, Brij-56 showed significantly higher NR intensity at this low CMC (0.005%) compared to Triton X-100. These findings provide further evidence of Brij-56's potential as an effective alternative to Triton X-100.

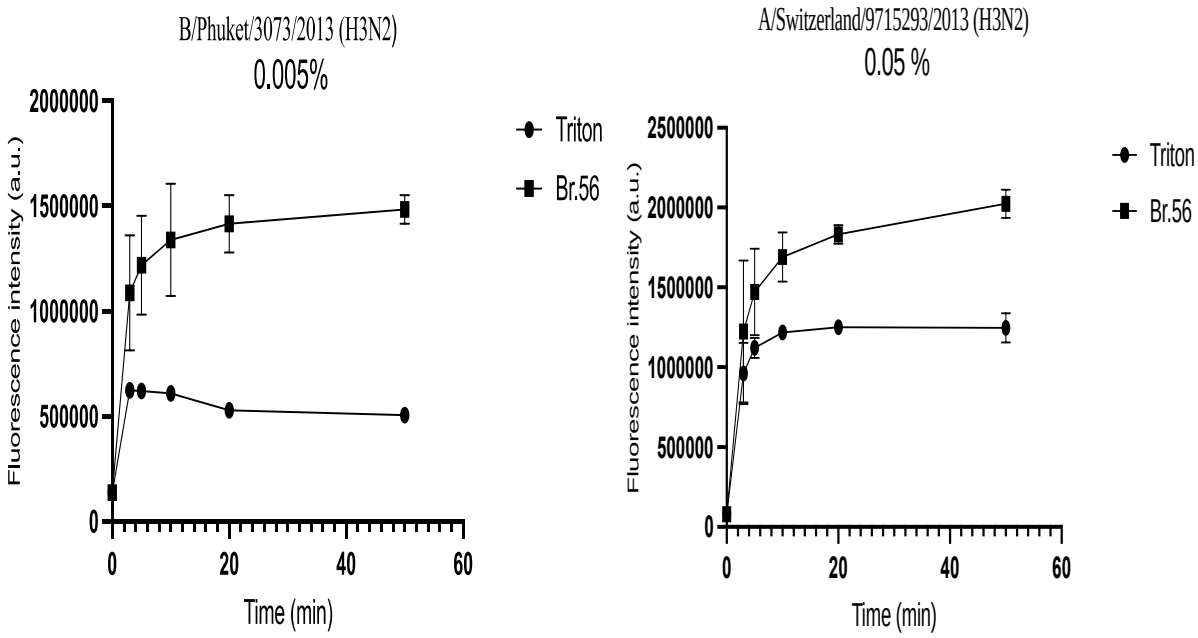

Fig. 3-4. The change of fluorescence intensity as a function of time after incubation of two influenza virus strains: A/Switzerland/9715293/2013 (H3N2) and B/Phuket/3073/2013 (H3N2) with Tx-100 and Brij-56.

NR is a fluorescent molecule categorized under a group of compounds that exhibit solvatochromism, wherein their emission spectrum varies depending on the polarity of their surrounding environment [31, 32]. In polar environments, the fluorescence intensity of NR decreases and shifts towards longer wavelengths. Our findings reveal that the observed red shift in the emission wavelength of NR from 595 nm to 639 nm indicates exposure to less hydrophobic surroundings. This may be due to the formation of micelles or the splitting of the virus, which generates a less hydrophobic environment than the lipid membranes of intact viruses (Fig. 3-5).

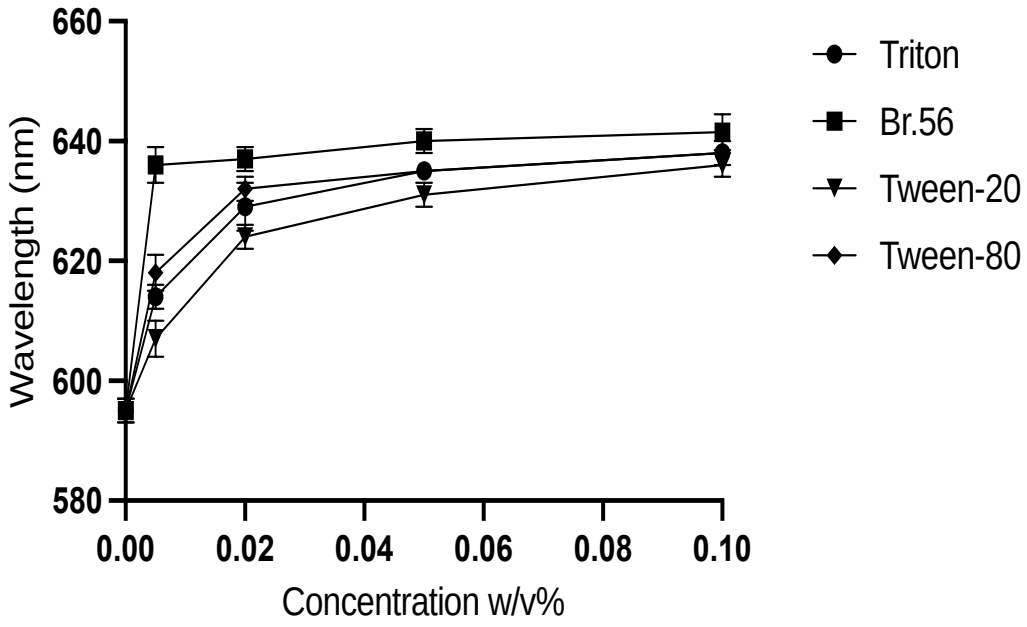

Fig. 3-5. The change of wavelength as a function of concentration after incubation of influenza virus (A/California/7/2009 (H1N1) with different surfactants.

The wavelength of all surfactants, except for Brij-56, consistently increases as concentration increases, plateauing at around 640 nm at 0.1% w/v. However, a red-shift from 595 nm to 639 nm was detected at the lowest concentration (0.005% w/v) of Brij-56. This can be attributed to

the comparatively lower CMC of Brij-56 (0.004% w/v) in contrast to the CMC values above 0.005% exhibited by the other surfactants.

In contrast to the other surfactants, Brij-56 exhibited no significant shifting of the wavelength even at low concentrations. This finding indicates that Brij-56 may form sufficient micelles or interact with virus particles to reduce the hydrophobicity of the surrounding environment. Despite the lower concentration utilised, Brij-56 interacts well with the virus particles, since its behaviour at low concentrations is similar to that of the other surfactants at greater concentrations.

It is clear that Brij-56 displays higher activity in comparison to the other surfactants based on the entire analysis of the NR studies, which includes intensity measurements and wavelength shifting. Brij-56 demonstrates higher NR intensity and wavelength shifting even at low concentration, indicating its enhanced performance. These findings suggest that Brij-56 holds promise as a viable alternative to Triton X-100. However, further investigations using additional techniques, such as morphological analysis of virus particles, are required to confirm the splitting activity of Brij-56.

3.2 Dynamic light scattering DLS

Dynamic Light Scattering (DLS) is a technique that is commonly utilised to determine the size distribution of particles that are dispersed in a solution [33]. Thus, DLS has gained extensive utilization in the examination of proteins, nucleic acids, and viruses [34, 35]. In this study, we utilised a Zetasizer Nano ZS (Malvern, United Kingdom) to measure the hydrodynamic radius of the influenza virus prior to and following the addition of Brij-56 and Triton-X to observe viral splitting using three different influenza strains: A/California/7/2009 (H1N1), A/Switzerland/9715293/2013 (H3N2), and B/Phuket/3073/2013 (H3N2) (Fig.3-6).

We have utilized the most concentrated surfactant solution (0.1%w/v) to ensure thorough disruption of the virus.

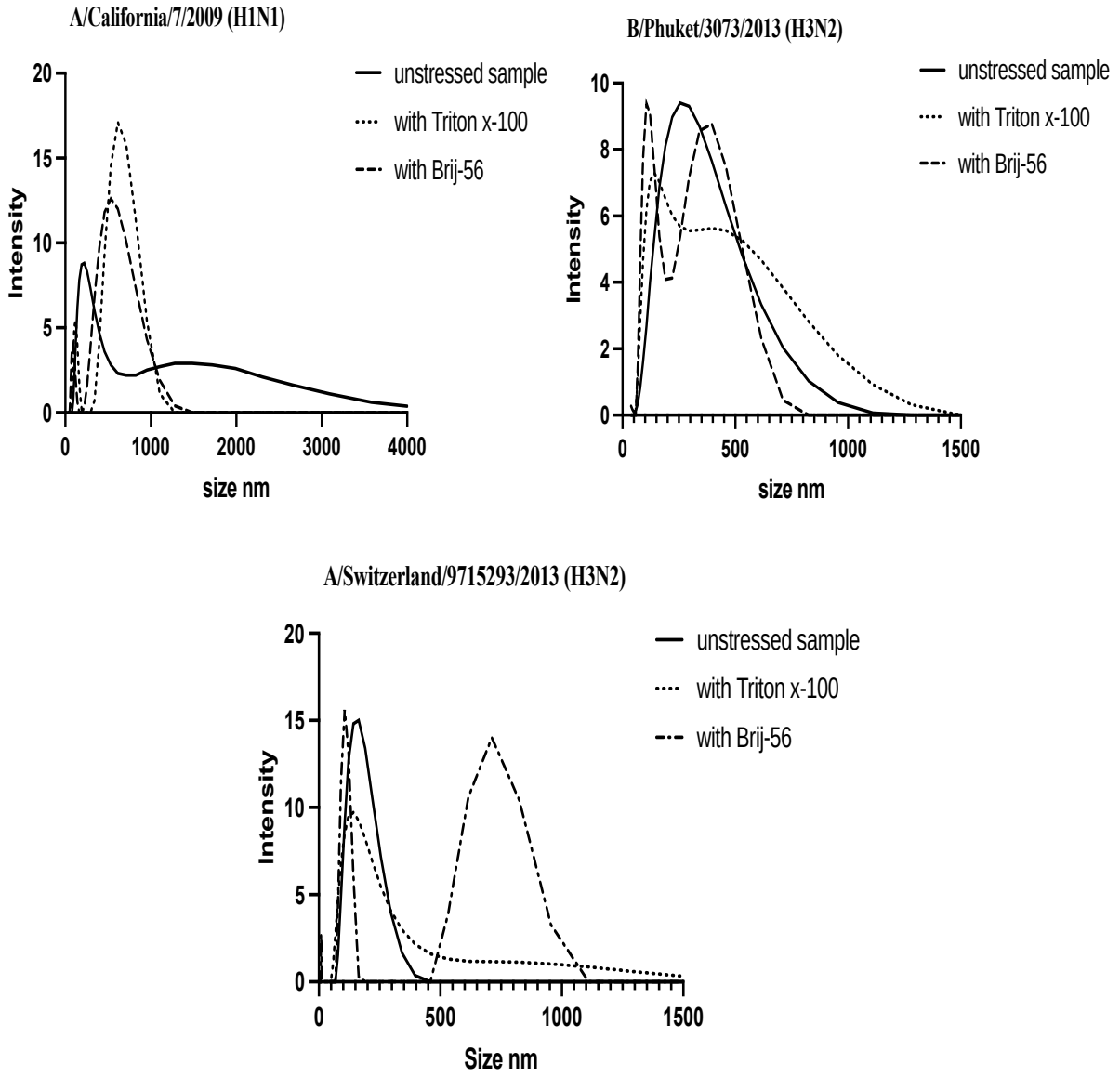

Fig. 3-6. The hydrodynamic size of different influenza virus strains using DLS. 0.1% w/v concentration for both Tritonx-100 and Brij-56 was used to observe the changing of the size as an indicator of the splitting after adding the surfactants.

Influenza A and B viruses exhibit either spherical or filamentous morphology, with the former measuring around 100 nm in diameter and the latter often exceeding this diameter in length [2, 36]. We have observed similar results for both strains in the absence of surfactants (unstressed

samples). Upon incubation with 0.1%w/v Tritonx-100 or Brij-56, we detected changes in the hydrodynamic size of the viruses across all strains. These changes manifested as various size populations of smaller or larger sizes, accompanied by a corresponding alteration in intensity. This phenomenon may be attributed to the presence of viral fragments resulting from the splitting process and/or their agglomeration. It's possible that viral proteins within the lipid membrane create the bigger clumps [20]. In contrast to Tritonx-100, Brij-56 exhibited more potent antiviral activity, particularly against the A/Switzerland/9715293/2013 (H3N2) and B/Phuket/3073/2013 (H3N2) strains, possibly due to its lower critical micelle concentration, which results in the formation of more micelles than Triton X-100.

3.3 Nanoparticle tracking analysis (NTA)

The Nanoparticle Tracking Analysis (NTA) is a method employed to determine the size of particles ranging from 30 to 1000 nm and quantify the concentration of particles within the range of 10E+7 to 10E+9 particles/ml, depending on the type of sample [37]. This technique has been extensively studied for measuring nanoparticles, drug delivery systems, and protein aggregates [38]. In the current study, NTA was employed to refine DLS measurements of the hydrodynamic diameter of viruses. The same viral samples used for size measurements via DLS were subjected to NTA analysis.

Our findings indicate that upon incubation with 0.1%w/v Tritonx-100 or Brij-56, the concentration of whole virus particles across all strains was altered, resulting in a decrease in the concentration of particles around 100 nm when compared to unstressed samples of the same size as an indication of the intact virus disruption (Fig. 3-7). Moreover, an increase in the number of particles that are either smaller or larger in size was observed as well.

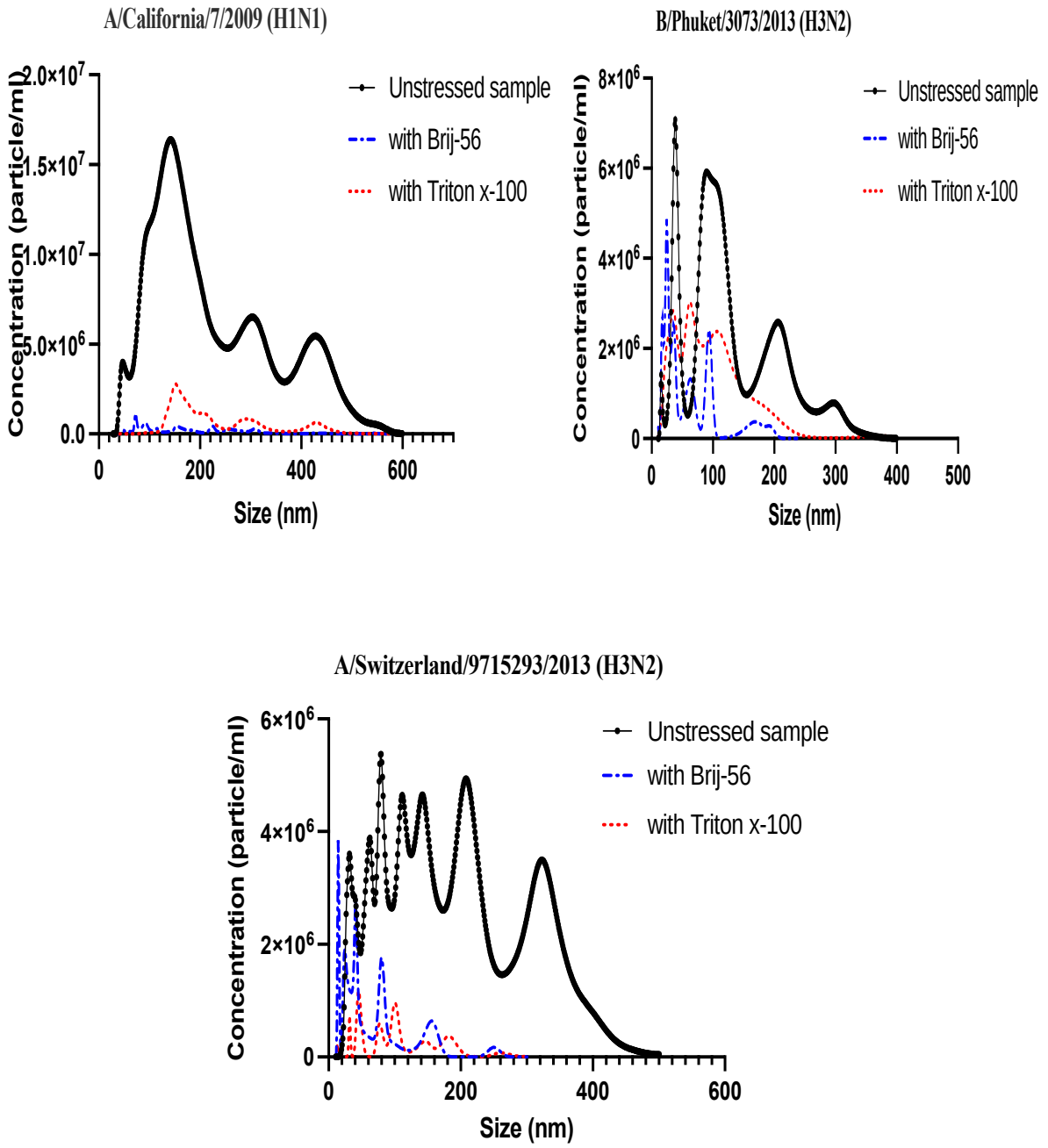

Fig. 3-7. The concentration (number) of virus particles/ml using NTA. 0.1% w/v concentration for both Tritonx-100 and Brij-56 was used to observe the changing of the concentration (number) of virus particles with their sizes as an indicator of the splitting after adding the surfactants.

These findings, similar to the DLS results, may be attributed to the creation of viral fragments resulting from the splitting process and/or their subsequent agglomeration. Additionally, the formation of larger clumps can be attributed to the viral proteins present within the lipid

membrane. Notably, samples incubated with Brij-56 exhibited lower particle concentrations than those treated with Triton X-100, which may be attributed to its superior ability to split when compared to Triton X-100.

3.4 Transition electron microscope (TEM)

The direct observation of viruses is only possible with imaging technology that offers nanometer-scale resolution, such as Transmission Electron Microscopy (TEM). Reference samples of whole viruses may comprise pleomorphic virions with a wide range of shapes and sizes, although the majority of these virions are typically spherical and measure approximately 100 nm in diameter [39-42]. In some instances, viruses may cluster in significant numbers, resulting in large agglomerates [43, 44].

As previously reported [1, 27], our observations indicate that the presence of surfactants significantly disrupts viruses when added as splitting agents (Fig.3-7). In untreated samples of all three influenza virus strains, viral particles with intact membrane proteins were observed. However, most viruses in a sample exposed to 0.1% w/v Triton X-100 or Brij-56 for 20 minutes exhibited disrupted membranes. While several viruses of the A/California/7/2009 (H1N1) strain were found to have retained their overall structure despite being treated with surfactants, the A/Switzerland/9715293/2013 (H3N2) and B/Phuket/3073/2013 (H3N2) strains only left behind amorphous clusters of virus particles after surfactant treatment.

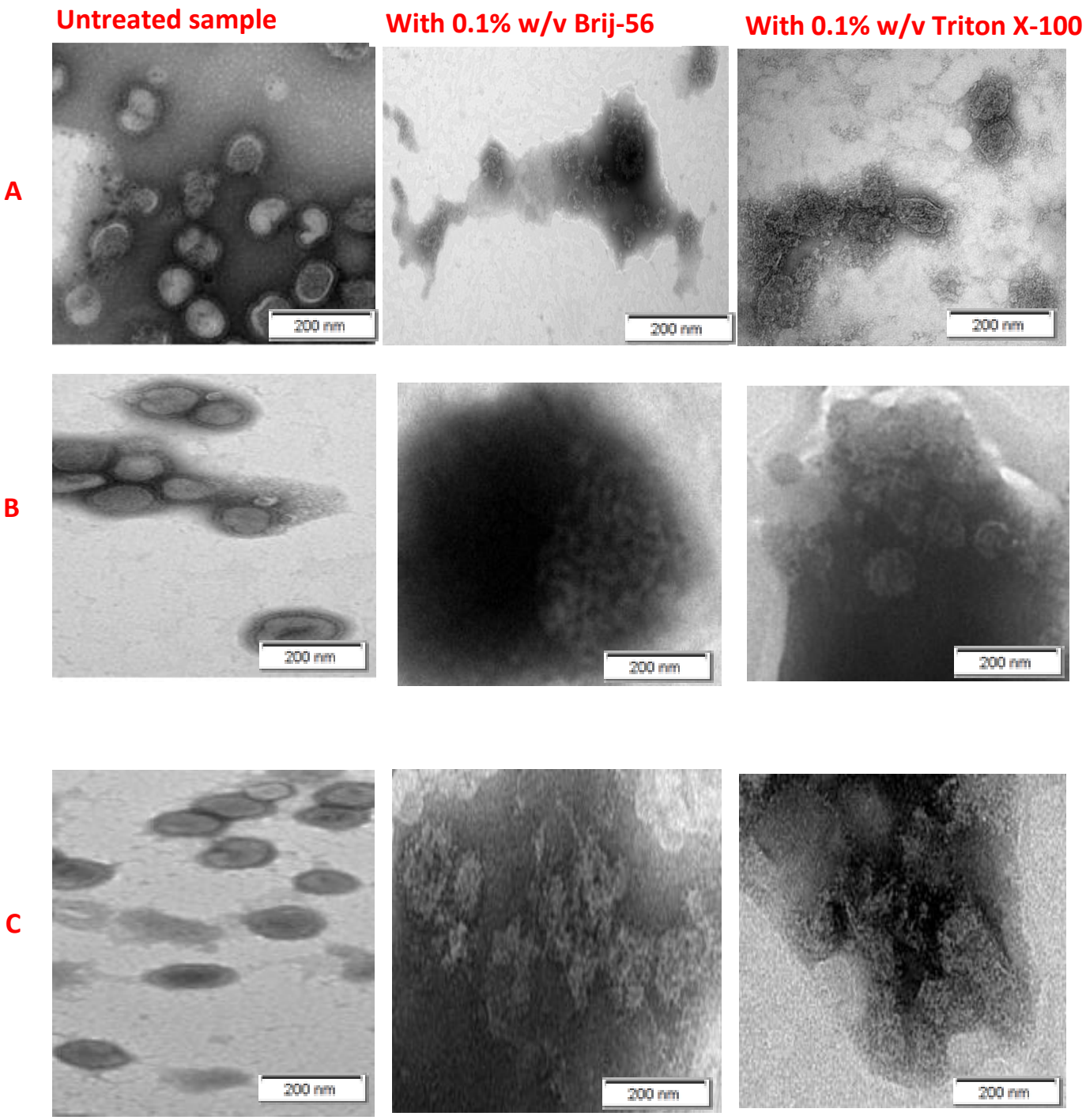

Fig. 3-7. Electron micrographs of negatively stained inactivated influenza viruses. (A) A/California/7/2009 (H1N1), (B) A/Switzerland/9715293/2013 (H3N2), (C) B/Phuket/3073/2013 (H3N2). Each strain has untreated sample, sample with 0.1% w/v Brij-56 or Triton x-100 after incubation for 20 minutes.

4. Conclusion

In our study aimed at addressing the limitations of Triton X-100 in the production of inactivated influenza vaccines, we thoroughly investigated several alternative surfactants, namely Tween-20, Tween-80, and Brij-56. We employed a comprehensive approach, utilizing fluorescence and light scattering techniques, as well as a transmission electron microscope, to evaluate the surfactants' interactions with the influenza virus and their potential for causing viral splitting. Three different strains of influenza virus were used throughout the study to guarantee the accuracy and breadth of our results: A/California/7/2009 (H1N1), A/Switzerland/9715293/2013 (H3N2), and B/Phuket/3073/2013 (H1N1).

Our in-depth investigation has led us to an important result. Without a phenyl ether moiety, Brij-56 stood out among the studied surfactants as a promising replacement for Triton X-100 in inactivated split-virus influenza vaccinations. According on our findings, Brij-56 strongly interacted with the influenza virus and caused viral cleavage. Results from fluorescence and light scattering methods corroborated these observations, showing that Brij-56 effectively interacted with virus particles. The use of a transmission electron microscope in our experiment allowed us to see the viral particles splitting as a result of contact with Brij-56. This additional confirmation further substantiated the suitability of Brij-56 as a surfactant for achieving viral splitting in the production of inactivated split-virus influenza vaccines.

Our extensive research demonstrates conclusively that Brij-56 is a suitable replacement for Triton X-100 as a splitting agent in the manufacture of inactivated split-virus influenza vaccines. Brij-56's promise as a viable alternative to Triton X-100 in vaccine manufacture is highlighted by the fact that it lacks a phenyl ether moiety and has been shown to interact with

the influenza virus and promote viral splitting. These results are important for the continuing research into making influenza vaccinations safer and more effective.

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CHAPTER 4

Enhancing the Stability of Whole Inactivated Influenza Virus for Novel Vaccine Formulations

CHAPTER 4- Chapter Overview

The following chapter is a full research article.

The current investigation is the first attempt to assess the ability of macrocycles to stabilize influenza virus. Although these compounds have found various pharmaceutical applications, their potential to improve influenza vaccine formulations has not been previously examined.

Abstract

In the prevention of infections, influenza vaccines find widespread utilization, with their common presentation being in the form of a liquid that necessitates cold storage and transportation. Nevertheless, the process of transportation may witness temperature fluctuations, thus exerting a detrimental impact on the stability of the vaccine and facilitating the aggregation of proteins. In order to tackle this concern, the present investigation delved into the potential of four distinct macrocycles (4-sulfocalix[4]arene, 4-sulfocalix[8]arene, cucurbit[5]uril, and cucurbit[7]uril) to serve as stabilizers for whole inactivated influenza virus. Through the utilization of fluorescence spectroscopy, dynamic light scattering (DLS), and transmission electron microscopy, the influence of these macrocycles on conformational alterations and the formation of aggregates at elevated temperatures was scrutinized. Among the four macrocycles scrutinized, sCX[4] demonstrated the most promising impact, as it enhanced conformational stability and reduced aggregation, thereby exhibiting its potential to stabilize the whole inactivated influenza virus. These significant findings signify the potential candidacy of sCX[4] in the advancement of future influenza vaccines.

1.Introduction

Influenza poses a recurring threat that results in significant morbidity and mortality worldwide, with an estimated one billion people affected annually. The economic burden is substantial, and the disease is responsible for a global death toll that ranges between 290,000 to 650,000 each year, with fluctuating rates [1]. Influenza vaccines are the primary method for preventing and controlling the disease and reducing its severity. To combat circulating and emerging strains, these vaccines are modified annually based on predictions by the World Health Organization [2]. The vast majority of liquid influenza vaccines necessitate refrigeration in order to preserve their effectiveness. Nonetheless, challenges pertaining to the transportation and storage of vaccines within the cold-chain system may give rise to temperature fluctuations or instances of elevated temperature exposure, thus jeopardizing the integrity of the vaccines [3].

Issues with the thermal stability of vaccines can lead to protein aggregation. Other factors, such as pH, added excipients, ionic strength, and protein concentration, can also contribute to this problem [4, 5]. Aggregation lowers or eliminates protein function because it alters the protein's natural structure [6-8]. In addition, the presence of aggregates might trigger adverse immunological responses, ranging from mild to severe and even lethal, including anaphylactic reactions.

The regulation of virus splitting during the production process using surfactants remains inadequate. Ensuring stability in the formulation of the complete virus poses a challenge due to variations in inherent viral stability among different viruses. Consequently, manufacturers necessitate approaches to address and reduce aggregation by employing stabilizers, modifying glycosylation, and introducing mutations in regions susceptible to aggregation [9, 10].

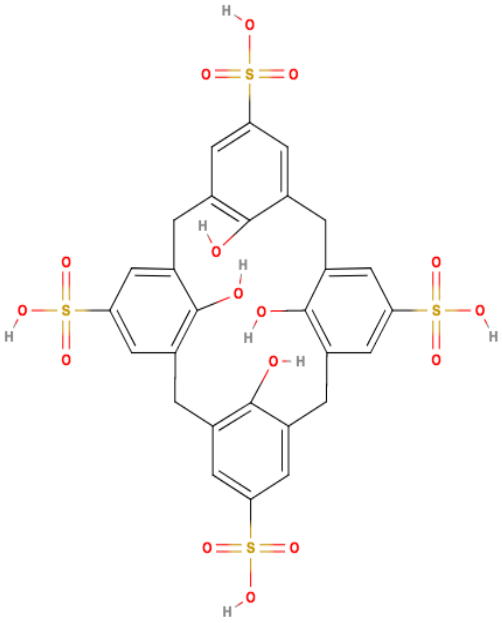
Influenza vaccine formulations commonly incorporate additives and stabilisers such as sucrose, Tween 80, and Triton X-100 to enhance stability and minimise aggregation [11, 12]. The objective of these stabilisers is to improve the protein's conformational or colloidal stability [13, 14], which is crucial in preventing aggregation. Conformational stability refers to the protein's ability to maintain its native state, while colloidal stability refers to the intermolecular forces between unfolded and folded proteins [15-17].

This study investigates the potential of macrocycles to stabilize influenza virus, representing the first evaluation of their effectiveness in this context (Fig. 4-1). Although macrocycles have various pharmaceutical applications, their role in enhancing influenza vaccine formulations has not been explored previously. Macrocycles possess advantageous characteristics such as enhanced stability under light, elevated temperatures, and acidic conditions, improved solubility and bioavailability, and the capability to encapsulate drugs by forming complexes through non-covalent interactions [18-20]. Furthermore, they demonstrate low toxicity and minimal immunogenicity, further highlighting their potential as beneficial components [18, 19].

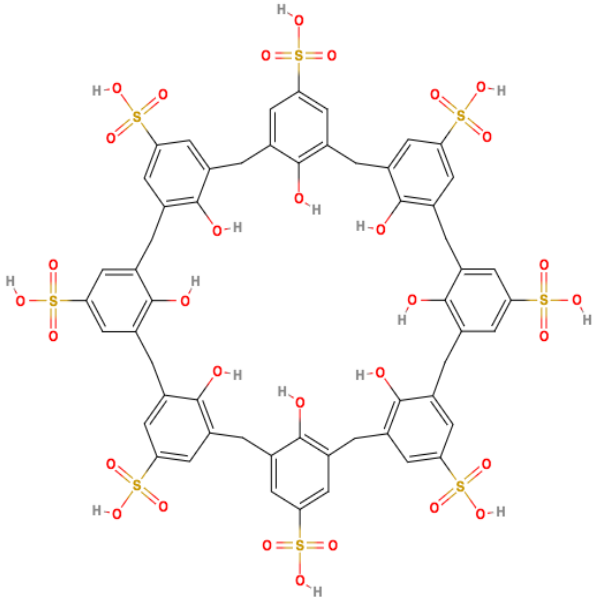
Calixarenes are one type of macrocycles that have an amphiphilic structure and can be chemically modified to develop various properties. Calixarenes exhibit structural diversity with their phenol units connected by methylene bridges in the ortho position [21]. These compounds can possess a varying number of aromatic units, ranging from 4 to 20. However, it is noteworthy that calixarenes consisting of 4, 5, 6, 7, and 8 aromatic units are the most prevalent members within this family [22]. The configuration of the macrocycle cavity, depicted in Fig. 4-1, is contingent upon the quantity of aromatic units present within the system. Calix[4]arenes offer the possibility of functionalization on either the upper or lower rim with various functional groups, including amides, imines, sulfur, azo, semicarbazone, alkyl groups, and

more. This diverse range of functionalisations gives rise to a broad array of macrocyclic compounds with distinct characteristics in terms of recognition, selectivity, solubility, and degree of hydrophobicity [23]. sCX[4] and sCX[8] are calixarenes with varying dimensions that feature a sulfonic group located para to the hydroxyl group. [18] (Fig.4-1). The significance of calix[n]arenes stems from their capacity to recognize and accommodate guest molecules within their cavity through non-covalent interactions. The strength of these interactions is also contingent upon the conformation adopted by the macrocycle [24].

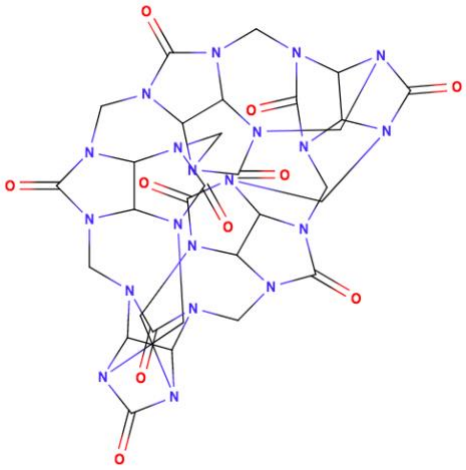
Cucurbiturils, a distinct class of macrocycles, have emerged as highly appealing macrocyclic hosts with significant applications in medicinal chemistry and chemical biology. During the first two decades of study on Cucurbiturils, the main focus was on their synthesis, structural characterisation, and their capacity to bind guest molecules. Nevertheless, as a result of progress in affordable synthesis and purifying techniques, together with a comprehensive comprehension of their characteristics, the research emphasis has shifted towards investigating potential uses for this interesting group of water-soluble macrocycles. The analysis of the structure of the Cucurbiturils has shown that they are macrocyclic compounds consisting of 5 to 10 glycoluril units, connected by two methylene bridges on both sides of the glycoluril segments. The arrangement of molecules in a cyclic manner leads to the creation of two symmetrical hydrophilic carbonyl portals, which exhibit partial negative charges, on each side. Additionally, there is a hydrophobic cavity with low polarity and polarizability [25]. Their appeal lies in their ability to form robust host-guest complexes with drugs, providing stabilization and efficient delivery of these therapeutic agents [19]. Furthermore, these macrocycles exhibit considerable potential in terms of being non-toxic and possessing high biocompatibility [26] . CB[5] and CB[7] are cucurbiturils composed of dissimilar quantities of glycoluril units connected by methylene linkages (Fig.4-2) [27].



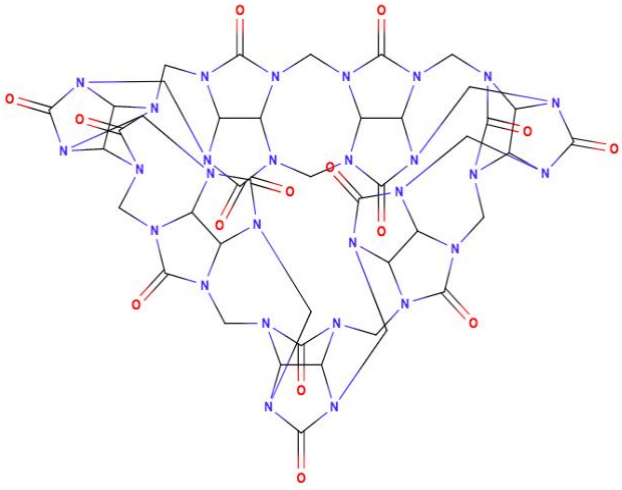
4-Sulfocalix[4]arene



4-Sulfocalix[8]arene



Cucurbit[5]uril



Cucurbit[7]uril

Figure 4-1: Chemical structures of 4 macrocycles that have been used in this study: 4-Sulfocalix[4]arene (sCX[4]), 4-Sulfocalix[8]arene (sCX[8]), Cucurbit[5]uril (CB[5]), and Cucurbit[7]uril (CB[7]).

The purpose of this study was to examine the effect of four different macrocycles on the conformational stability of complete inactivated influenza virus: sCX[4], sCX[8], CB[5], and CB[7].

The choice of these particular macrocycles is intended to include two separate classifications, so facilitating a full evaluation of their efficacy as stabilisers and possible variations in their effects. Moreover, it has been recorded that these specific macrocyclic compounds provide benefits in comparison to other alternatives. These advantages include a streamlined synthesis procedure, the retention of the drug's amorphous structure, and minimal effects on surface area and pore size distribution. These characteristics promote efficient processing and the creation of strong formulations.

In contrast to the complexity of influenza vaccine strains, which encompass multiple strains and excipients, preliminary studies employed whole inactivated influenza virus for a more focused analysis. Finding the best macrocycle to use as a stabiliser for whole inactivated influenza virus was the key focus. This was done by measuring conformational changes as a function of increasing temperature using tryptophan fluorescence assays. These tests were critical for evaluating the stability of the influenza virus in the presence of different macrocycles at varying temperatures. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) were used to get more insight into the influenza virus's dynamic particle size and shape using the most promising stabiliser discovered via fluorescence investigations. Influenza A/Switzerland/9715293/2013 (H3N2) and B/Phuket/3073/2013 (H3N2) were both studied to see how they were affected by these macrocycles.

2.Materials and Methods

2.1 Materials

All influenza virus samples were donated by Sanofi-Pasteur (Pennsylvania, USA). The strains studied were B/Phuket/3073/2013 (H3N2) and A/California/7/2009 (H1N). Purchases of

sCX[4], CB[5], and CB[7] were made from Sigma Aldrich (USA), whereas purchases of sCX[8] were made from Combi-Blocks (USA). Copper TEM grids coated with carbon and Formvar were acquired from ProSciTech (Queensland, AUS). All spectroscopic investigations used a Hellma quartz microcuvette from New York, USA. The information was analyzed using GraphPad Prism 9.

2.2 Preparation of samples

An influenza virus sample was diluted with distilled water and well mixed to create a 10% v/v stock solution of complete inactivated influenza virus. Stock solutions of sCX[4], sCX[8], CB[5], and CB[7] were prepared at a concentration of 2.5 mM in distilled water and allowed to dissolve completely overnight before utilization. Dilution of the samples was carried out using deionized water. To prevent aggregation and/or dissociation of formed aggregates, gentle pipetting was employed for mixing. This stage is crucial for the purpose of isolating temperature-induced aggregation or viral dissociation, while eliminating any potential impact from intense mixing.

2.3 Fluorescence emission spectroscopy

The fluorescence emission spectrum of Tryptophan was measured in the range of 290 to 400 nm, with excitation at 280 nm, using a Horiba Jobin Yvon Fluorolog FL-322 instrument (New Jersey, USA) equipped with 4 nm slits and an integration time of 0.15 seconds. Incubation of influenza virus samples with varying concentrations of sCX[4], sCX[8], CB[5], and CB[7] was carried out for a duration of 30 minutes. Subsequently, the samples were subjected to a temperature ramp from 25°C to 90°C, with each temperature point being equilibrated for 30 seconds prior to fluorescence measurement. A 50 μL quartz micro-cuvette from Hellma (New

York, USA) was used for fluorescence measurements. Each sample was analyzed in triplicate before further analysis using GraphPad Prism 9 software.

2.4 Dynamic light scattering (DLS)

The Zetasizer Nano ZS instrument, manufactured by Malvern (United Kingdom), was used to examine the distributions of monomer sizes and aggregation in samples of influenza virus. In the experiment, 1.5 mL samples of 10% v/v whole inactivated influenza virus were subjected to temperatures of 25 °C and 65 °C for 5 minutes with and without the addition of 1.07 mM sCX[4]. Whole inactivated influenza virus in water was given a refractive index of 1.45 and an absorption value of 0.001. Disposable plastic cuvettes were employed for sample analysis, maintaining a constant temperature of 25˚C throughout the measurements.

2.5 Transition electron microscope (TEM)

TEM images of influenza viruses were captured using a JEOL JEM-1400 instrument from Tokyo, Japan. Three samples of each influenza strain's whole virus were made. Two samples were treated with 1.07 mM sCX[4] and one acted as a control before being put on TEM grids. To facilitate imaging, negative staining was conducted using a 2% w/v solution of ammonium molybdate in water, with a duration of one minute. Subsequently, the grids were gently dried using filter paper and left to naturally dry before being subjected to imaging.

3. Results and discussion

3.1 Fluorescence emission spectroscopy

The tryptophan fluorescence was measured to evaluate the conformational changes in the protein structure of the influenza virus in the presence of different macrocycles. In the control influenza virus sample, a distinct decrease in the maximum fluorescence wavelength was

observed, starting from 25˚C and continuing until 30˚C. This decline was followed by a gradual decrease in the wavelength, reaching its lowest point compared to the samples treated with sCX[4] (Fig.4-1). Notably, this decline was most pronounced, with a reduction from approximately 323 nm at 25˚C to around 302 nm at 90˚C. The detected shift towards shorter wavelengths implies the existence of alterations in the wavelength, leading to conformational changes that cause protein unfolding and the formation of aggregates. These observations are consistent with prior research findings [28-30].

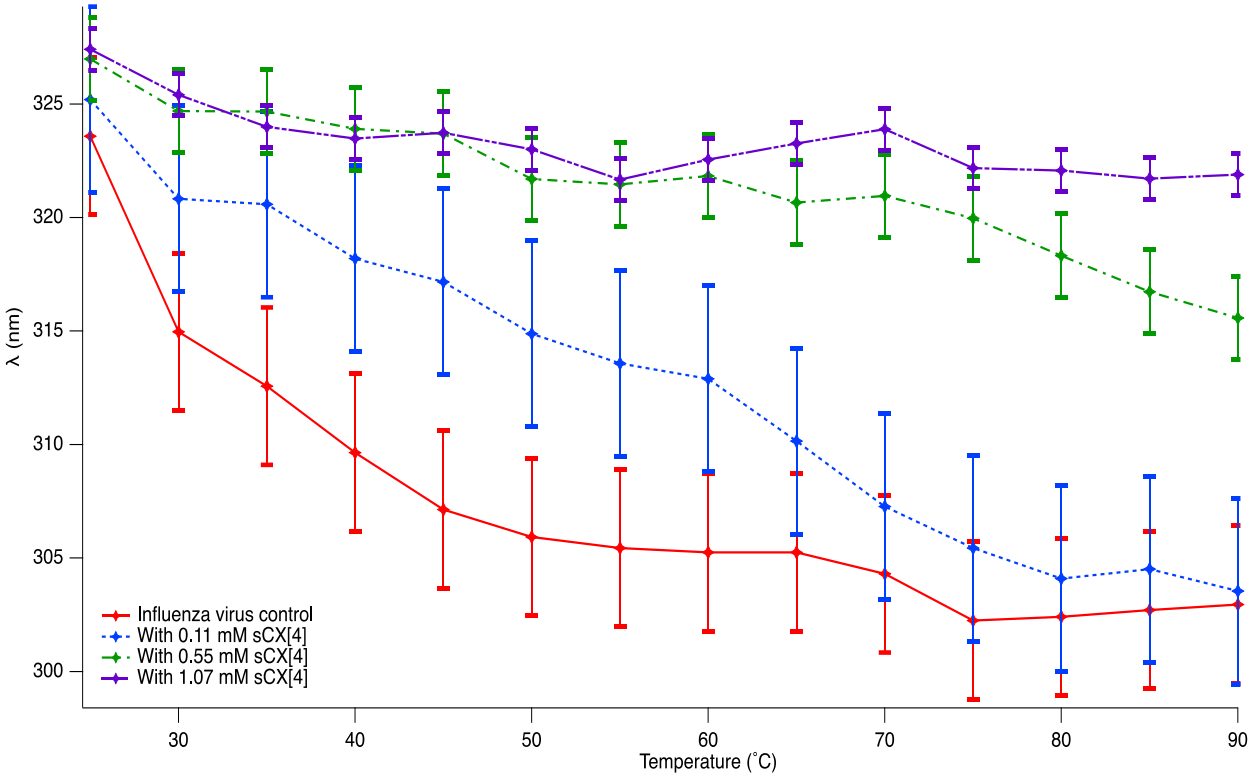


Figure 4-2: Fluorescence emission spectra of tryptophan at three different doses of sCX[4] (0.11 mM, 0.55 mM, and 1.07 mM) from whole, inactivated influenza virus samples.

Maximum tryptophan fluorescence wavelength changes reflect a general trend towards a more hydrophobic atmosphere. The sample with the lowest concentration of sCX[4] at 30 °C did not show the sharp drop seen in the control sample (Fig. 4-2). Fluorescence wavelength

decreased with temperature; however this trend was not statistically different from the control sample. Until around 60 °C, the fluorescence peak wavelength was essentially stable at 0.55 mM and 1.07 mM of sCX[4]. At 90 C, there was a noticeable gap between the 0.55 mM and 1.07 mM sCX[4] concentrations, and the 0.55 mM sCX[4] sample shown additional reductions thereafter (Fig. 4-2). At 25 degrees Celsius, 0.55 and 1.07 millimolar sCX[4] caused a change in the maximal tryptophan emission wavelength to 315 and 321 nanometers, respectively (Fig. 4-2). In comparison to the control sample, this change was less dramatic, suggesting less conformational change at temperatures over 60 °C . The overall structure of the influenza virus was better protected against heat-induced breakdown when sCX[4] was used at these concentrations, which is surprising given the tendency of proteins to congregate beyond their melting temperatures. These findings point to the possibility that a 1.07 mM concentration of sCX[4] might improve the stability of the influenza virus in vaccine formulations.

Our research revealed that the fluorescence peak wavelength for 0.11 mM sCX[8] combined with whole inactivated influenza virus was about 325 nm at 25 °C and around 306 nm at 90 °C (Fig. 4-2 and 4-3). Whole inactivated influenza virus with 0.55 mM sCX[8] showed impressively consistent maximum fluorescence wavelength throughout temperature ranges, with just slight changes at 60 °C and 70 °C . (Fig. 4-3). This was similar to the results obtained from a sample containing 0.55 mM sCX[4], but on a somewhat different scale, since the peak wavelength at 25 °C was about 313 nm rather than 326 nm (Fig. 4-2 and 4-3). The fluorescence peak wavelength in the 1.07 mM sCX[8] sample increased at first, but then settled at 35 °C. (Fig. 4-3). The presence of tryptophan in a more hydrophilic environment resulted in a red shift in the fluorescence wavelength, suggesting the development of unfolded proteins. As the error bars for both the experimental and control groups overlapped at some point at temperatures over 30 °C , it can be concluded that no sCX[8] concentration significantly deviated from the control.

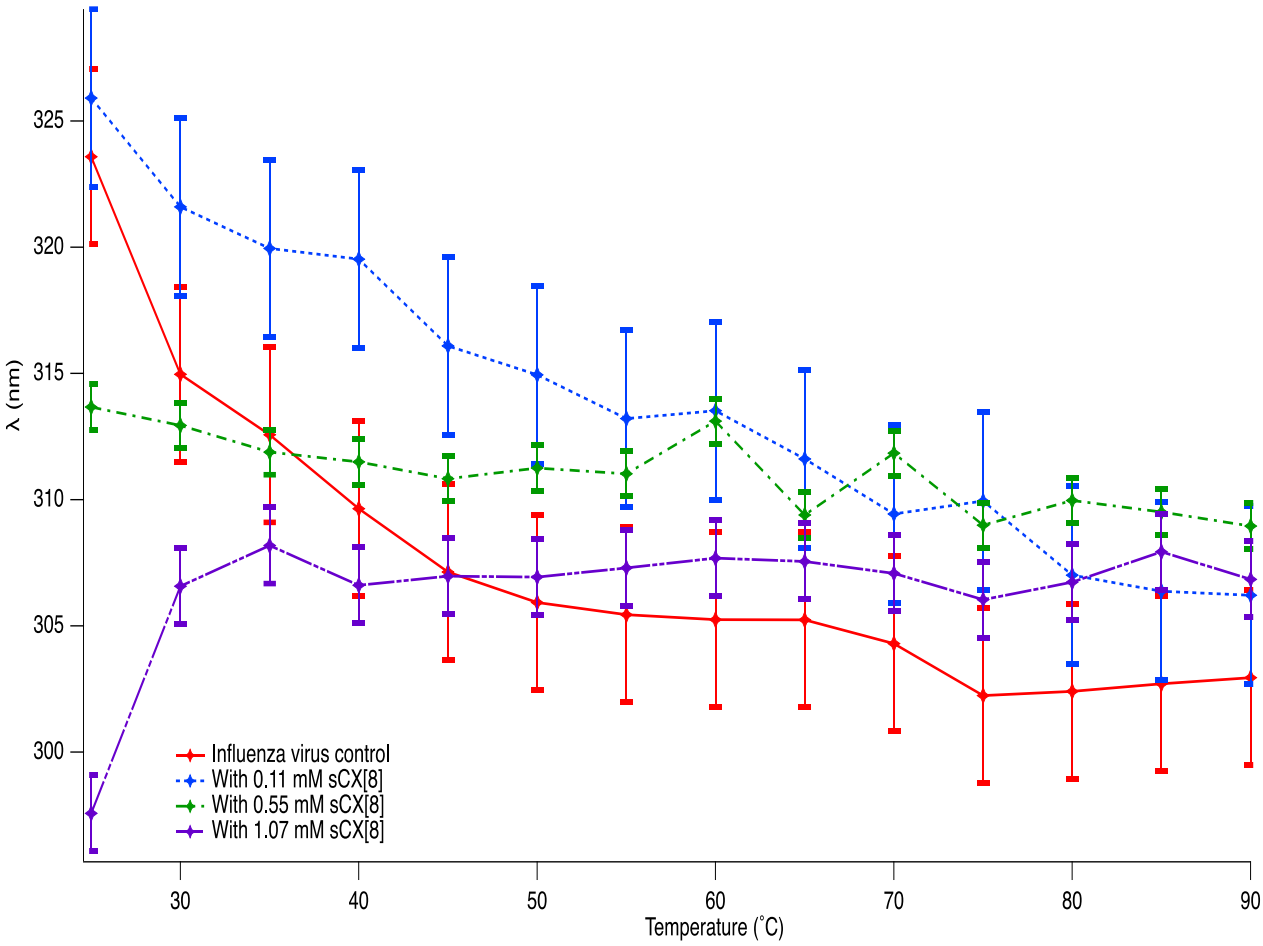


Figure 4-3: Fluorescence emission spectra of tryptophan at three different doses of sCX[8] (0.11 mM, 0.55 mM, and 1.07 mM) from whole, inactivated influenza virus samples.

The capability of a given macrocycle to form a complex with a certain guest molecule depends on its inclusion qualities. To attach to their respective guest molecules, sCX[4] and sCX[8] may, for instance, vary their conformational cavity's mobility [31]. When detecting guest molecules to bind, calixarenes mostly generate stacking interactions [6, 32, 33]. This quality allows them to attach to hydrophobic regions exposed at the protein surface, such as tryptophan. Nevertheless, earlier studies have shown that binding effects may be reversed when macrocycle size climbs to sCX[8], likely owing to steric blockage with the guest molecule's binding pocket [12, 34]. This may explain why sCX[8] has little effect on the conformational stability of the influenza virus .

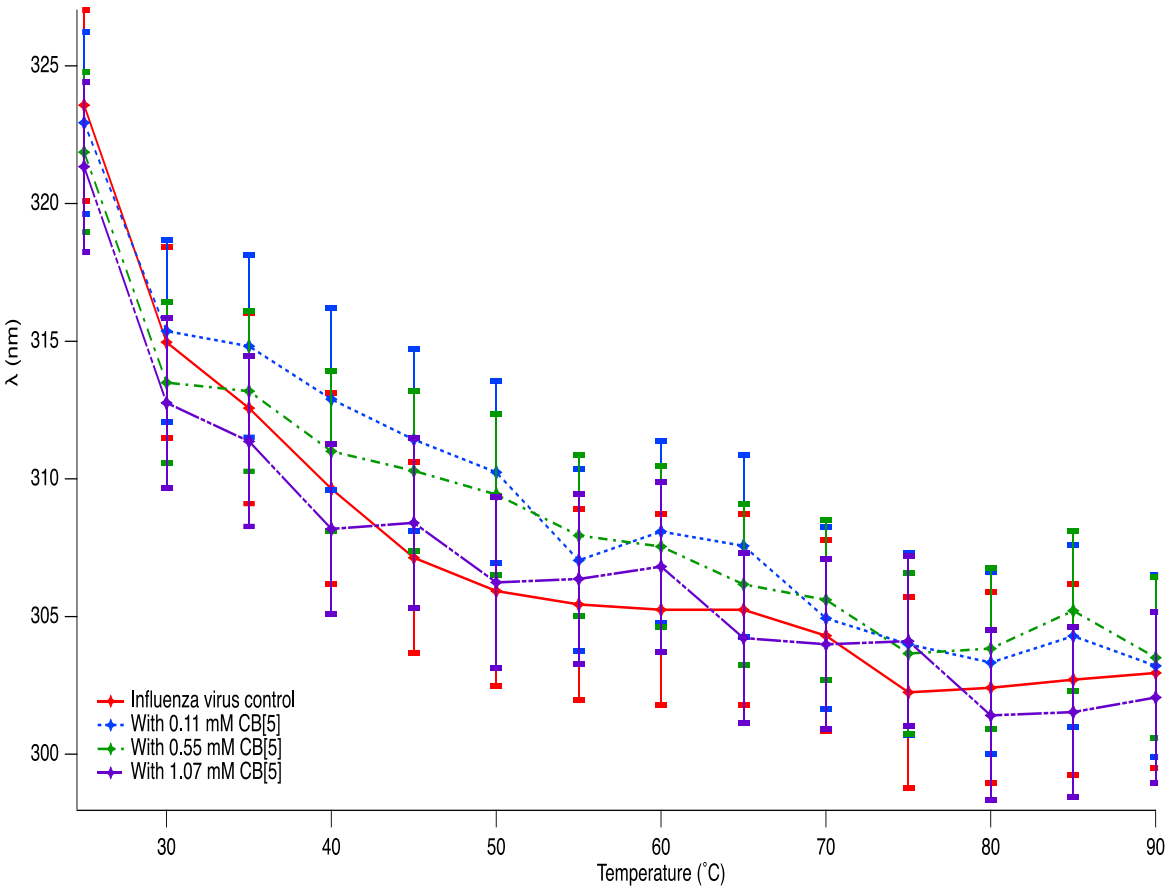


Figure 4-4: Fluorescence emission spectra of tryptophan at three different doses of CB[5] (0.11 mM, 0.55 mM, and 1.07 mM) from whole, inactivated influenza virus samples.

At 25 °C, there is little difference between entire inactivated influenza virus with CB[5] and CB[7] in terms of maximum wavelength of emission (Fig. 4-4 and 4-5). Furthermore, the maximum tryptophan emission wavelength consistently decreases across all doses of CB[5] and CB[7] with influenza virus, similar to the trend found in the control sample (Fig. 4-4 and 4-5). Maximum tryptophan emission wavelength changes from 25 to 90 degrees Celsius similarly between CB[5], CB[7], and control. These findings suggest that cucurbiturils may have a modest effect in delaying temperature-induced conformational changes associated with protein unfolding or aggregation. Maximum peak wavelength was also not significantly different between the control group and any concentration of CB[5] or CB[7] combined with

the whole inactivated influenza virus (Fig. 4-4 and 4-5). This suggests that cucurbiturils may not significantly enhance the stability of influenza vaccines during long-term storage or cold-chain transportation.

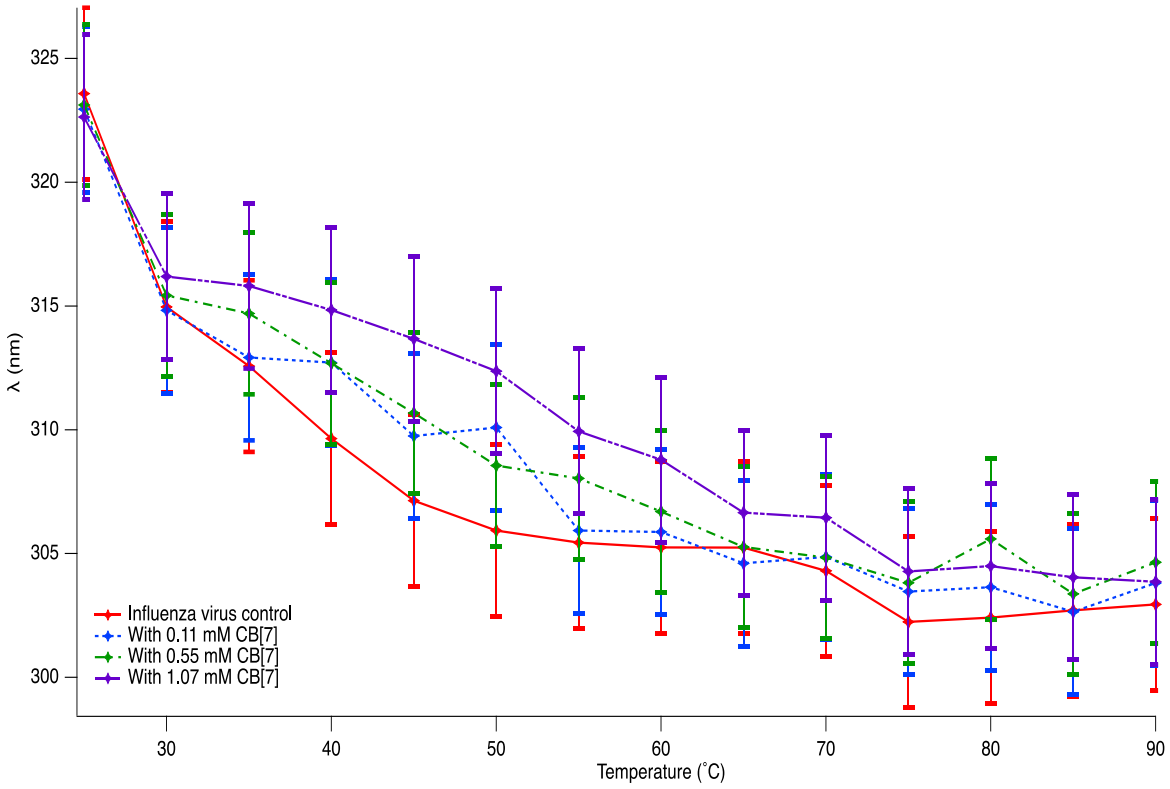


Figure 4-5: Fluorescence emission spectra of tryptophan at three different doses of CB[7] (0.11 mM, 0.55 mM, and 1.07 mM) from whole, inactivated influenza virus samples.

It is vital to recognise that cucurbiturils use both ion-dipole and hydrophobic contacts when comparing the ability of CB[5] and CB[7] to create connections with guest molecules to that of calixarenes [31, 35, 36]. In addition, cucurbiturils are able to build complexes in a more stable orientation because to their rigid structure, which is the consequence of their symmetrical nature and the consistent cavity openings this creates [31]. As compared to calixarenes, cucurbiturils have a number of distinguishing features that may explain why they have an effect on the structural changes of entire inactivated influenza virus when exposed to high temperatures.

Changes in maximum emission wavelength seen in 1.07 mM samples may be attributable to the naturally occuring fluorescence of macrocycles, in particular sCX[8] and sCX[4] [37]. As sCX[4] showed the most striking outcome, it is unclear whether it is related to the protein structure in general or to the whole inactivated influenza virus.

Seasonal influenza vaccines typically contain inactivated influenza viruses from both influenza A and B strains. As part of the development process for potential new additives, it is important to study their effects on both strains to ensure the widest possible effectiveness. To this end, the most promising macrocycles were identified through the above rigorous testing process, and the optimal concentration (1.07 mM sCX[4]) was subsequently tested on the influenza B virus strain B/Phuket/3073/2013 (H3N2) (Fig.4-6).

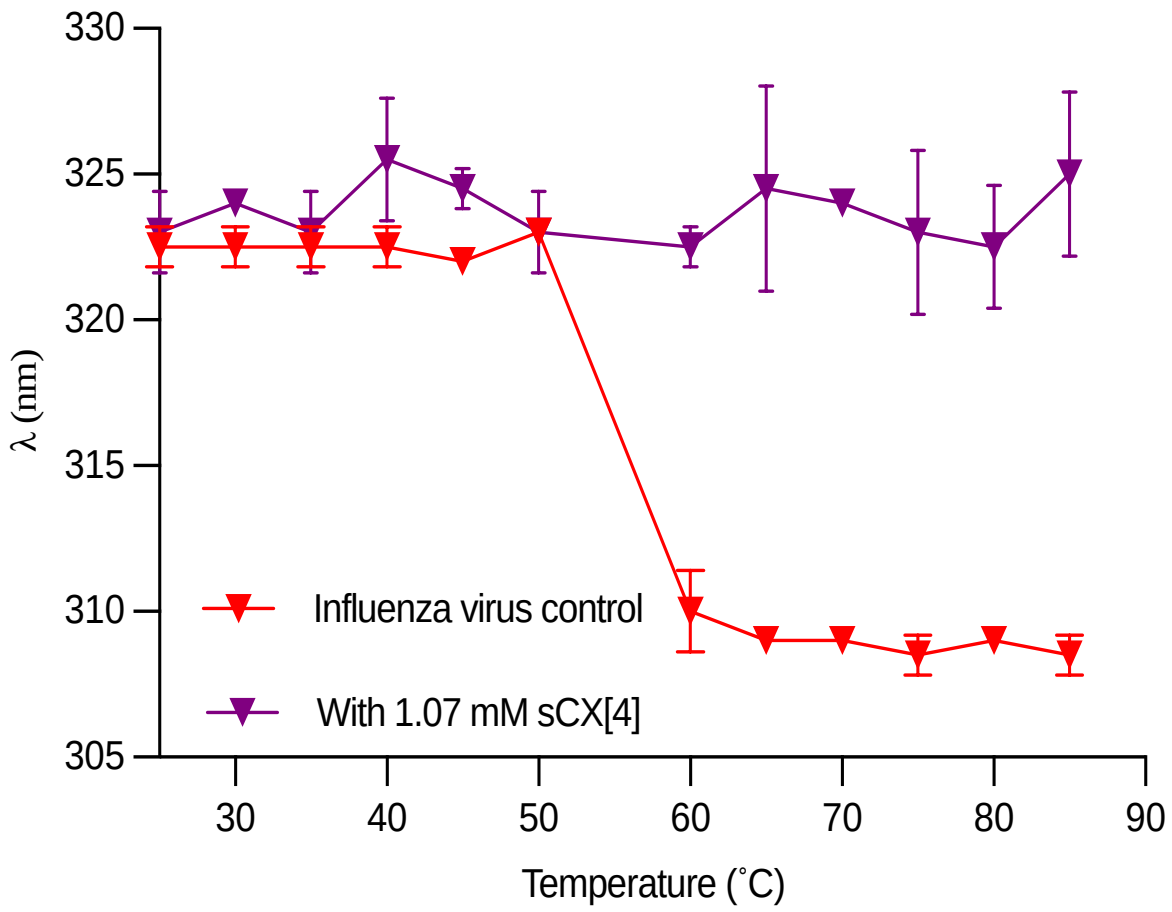


Figure 4-6: Tryptophan maximum fluorescence emission spectra of whole inactivated influenza B virus strain B/Phuket/3073/2013 (H3N2) samples with/without 1.07 mM sCX[4].

The analyzed control B/Phuket/3073/2013 (H3N2) influenza virus sample demonstrated consistent maximum fluorescence wavelength values between 25˚C and 50˚C, but a sudden decrease was observed at 60˚C (Fig.4-6). Thereafter, no significant alterations in the maximum fluorescence wavelength were detected beyond 60˚C. The observed decline in maximum fluorescence wavelength was statistically significant, indicating a notable reduction from approximately 323 nm at 25˚C to around 310 nm at 60˚C. The identified blue shift in wavelength implies the likelihood of conformational alterations resulting in protein unfolding and the formation of aggregates, which aligns with prior research findings [28-30]. Moreover, While the blue shift in wavelength observed in the fluorescence emission spectrum can be attributed to conformational changes and protein unfolding, another contributing factor could be the decrease in tryptophan intensity with increasing temperature. As the temperature increases, the fluorescence intensity of tryptophan decreases until it falls below the maximum intensity of tyrosine, which has an emission wavelength of around 310 nm. This might cause a blue shift in the fluorescence emission spectrum, shifting towards lower wavelengths.

As compared to the control sample, the solution containing 1.07 mM of sCX[4] maintained a relatively steady fluorescence peak wavelength throughout a broad temperature range, showing that the protein's structural integrity was maintained (Fig.4-6). This might be because the protein structure is stabilised upon sCX[4] binding, making conformational changes and protein unfolding less likely. To further preserve the fluorescence intensity and peak wavelength, it is probable that sCX[4] binds with tryptophan residues in the protein, protecting them from the effects of rising temperature. This is in contrast to the untreated control sample, where a blue shift in the fluorescence emission spectrum was detected due to structural changes and protein unfolding due to the lack of stabilising components.

3.2 Dynamic light scattering (DLS)

One typical method for determining the particle size distribution in a solution is dynamic light scattering (DLS) [38]. DLS's ability to examine the hydrodynamic behaviour of proteins, nucleic acids, and viruses has been particularly beneficial due to the information it can offer on both size and aggregation [39, 40].

We used a Zetasizer Nano ZS (Malvern, United Kingdom) to compare the hydrodynamic radius of the virus with and without the stabiliser after straining the sample at 60 °C in order to better understand the impact of sCX[4] on the stability of the influenza virus. We can learn more about how the presence of sCX affects the size of the virus particles by calculating the hydrodynamic radius[4] (Fig.4-7).

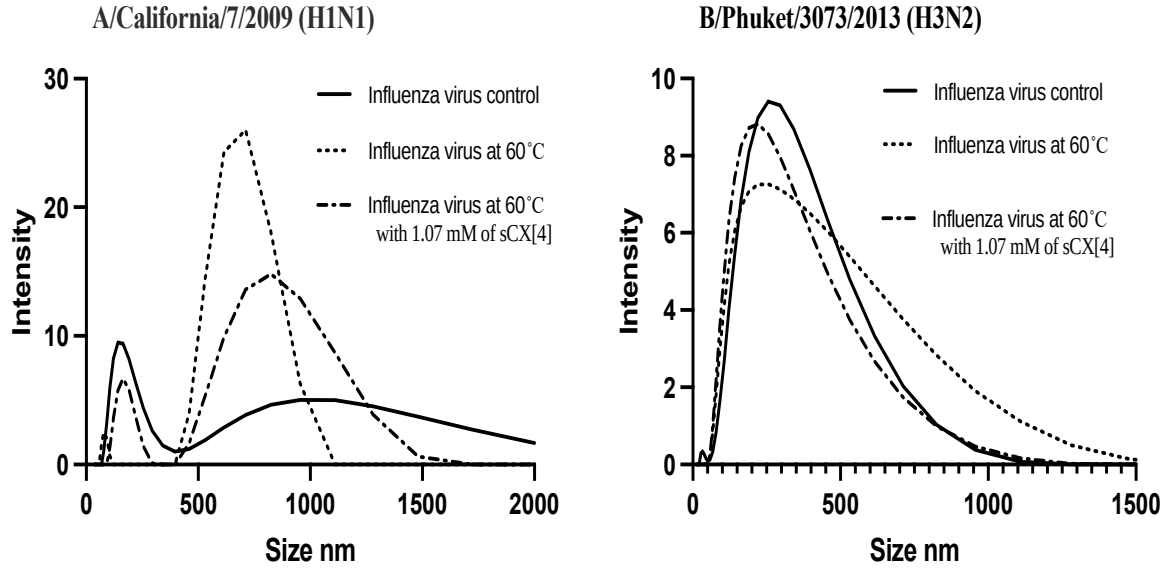


Fig. 4-7. The hydrodynamic size of different influenza virus strains using DLS. A control unstressed samples of A/California/7/2009 (H1N) and B/Phuket/3073/2013 (H3N2) were compared to samples with 1.07 mM of sCX[4] at 60˚C.

Since heat can so efficiently destroy the structure and function of viral particles, it is a frequent approach for inactivating viruses. The instability of the viral membrane at high temperatures may cause it to get damaged or rupture, making the virus unable to infect host cells [41, 42]. In terms of shape, influenza A and B viruses may be either spherical (about 100 nm in diameter) or filamentous (often longer than 100 nm), depending on the strain [43, 44]. Our study investigated two strains - A/California/7/2009 (H1N1), and B/Phuket/3073/2013 (H3N2) - and observed similar results in the control unstressed samples (Fig.4-7). At 60 degrees Celsius, the A/California/7/2009 (H1N1) strain's population shrank to below 100 nm in size, whereas the B/Phuket/3073/2013 (H3N2) strain's population shrank to below 100 nm in size and lost intensity.

The Zetasizer analysis's finding of a trend towards bigger size distributions suggests that an increase in the hydrodynamic radius of influenza virus populations may be connected to the consequences of temperature-induced changes in viral structure. Viral membranes may become unstable at elevated temperatures [41, 42], leading to particle aggregation and the formation of bigger structures that increase the hydrodynamic radius of the virus. The establishment of bigger size populations in the sample may also be influenced by protein denaturation and aggregation [45]. As a result, the Zetasizer analysis's finding of a trend towards bigger size distributions after subjecting the sample to stress at 60 °C may be suggestive of the aggregation or structural changes in the influenza virus particles brought on by the elevated temperature.

Hydrodynamic radius of influenza virus populations remained essentially stable following incubation with 1.07 mM of sCX[4] in both strains, and size distributions displayed patterns comparable to control unstressed samples, suggesting that sCX[4] may have a protective effect against temperature-induced aggregation and structural alterations. Possible mechanisms for the stabilising effect of sCX[4] include binding to the viral membrane to prevent denaturation

and aggregation, and interaction with viral proteins to do the same. To further stabilise the viral structure, sCX[4] may also work as a crowding agent, decreasing the available area for aggregation.

3.4 Transition electron microscope (TEM)

Only with imaging technologies of nanometer-scale resolution, such TEM, is it feasible to directly see viruses. Although pleomorphic virions of all shapes and sizes are possible in whole-virus reference samples, the vast majority of virions are typically spherical and measure about 100 nm in diameter [46-49]. Viruses have been shown to aggregate in high quantities under some situations [50, 51].

Here, the electron microscope was employed as a complimentary technique to the Zetasizer Nano ZS to offer more insightful data on how sCX[4] affects the stability of the influenza virus. Electron microscopy provides supplemental data to further understand the influence of the stabiliser on the virus under stress circumstances like heat due to its higher resolution imaging, which permits more precise observations of structural changes in the virus. We used electron microscopy to compare stressed and unstressed samples of both influenza virus strains and found that incubation of the virus at 60 °C without the stabiliser caused the virus to undergo structural alterations (Fig.4-8). It is possible that the proteins in the influenza virus denature during exposure to high temperatures, changing their structure in the process [52]. This may cause disruptions in the structure of the viral particles, as shown by electron microscopy (Fig.4-8). The influenza virus's lipid envelope may potentially be affected by temperature variations. Possible modifications to the virus's outer envelope due to heat-induced membrane rupture or fluidity changes [53].

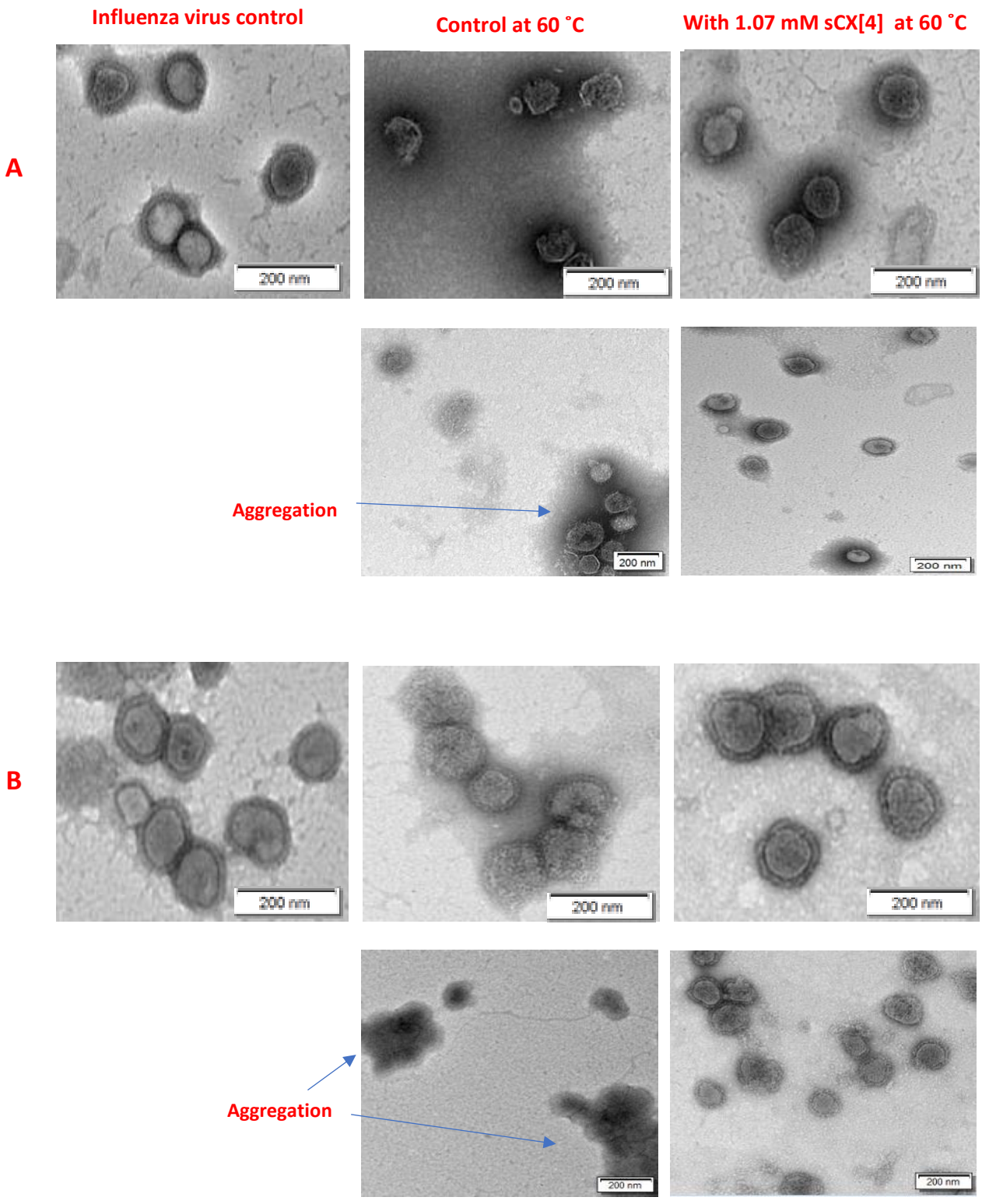


Fig. 4-8. Electron micrographs of negatively stained inactivated influenza viruses. (A) A/California/7/2009 (H1N1), (B) B/Phuket/3073/2013 (H3N2). Each strain has untreated sample, control sample at 60 ˚C, and sample with 1.07 mM sCX[4] at 60 ˚C.

Due to the stabilising properties of the sCX[4] molecule, we saw some structural integrity preserved in the influenza virus sample when it was incubated with 1.07 mM of the stabiliser at the same temperature (Fig.4-8). The potential stabilizing mechanism of sCX[4] could be attributed to its ability to bind to the viral membrane and prevent destabilization and aggregation (Fig.4-8), as well as its effect to prevent protein denaturation by inhibiting protein-protein interaction[54]. Furthermore, sCX[4] may act as a crowding agent, reducing the available space for aggregation and promoting a more stable structure of the virus.

4. Conclusion

With the use of fluorescence, dynamic light scattering (DLS), and transmission electron microscopy (TEM), we were able to deduce that sCX[4] had two effects on complete inactivated influenza virus: (1) increased conformational stability, and (2) decreased aggregation formation. In order to assure the broadest potential efficacy, it was tested on two distinct types of influenza. Further research is needed to determine the synergistic effects and overall appropriateness of various excipients widely used in influenza vaccine formulations. Interestingly, sCX[4] showed the most promise as a stabiliser option for influenza vaccinations among the macrocycles examined.

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CHAPTER 5

Enhancing the Stability of COVID-19 vaccines

CHAPTER 5- Chapter Overview

This research chapter provides a comprehensive investigation into the thermal stability and aggregation behavior of COVID-19 vaccine formulations, with a particular focus on the effects of the stabiliser sCX[4]. The findings presented in this study are of great significance to the research field as they shed light on the importance of stabilisers in maintaining the structural integrity of vaccines, especially under elevated temperature conditions. By utilising advanced techniques such as fluorescence spectroscopy, light scattering, and electron microscopy, this research offers valuable insights into the mechanisms of vaccine stability and the potential benefits of incorporating sCX[4] as a stabiliser. This work contributes to the ongoing efforts to optimise vaccine formulations, enhance their shelf life, and ensure their effectiveness in combating the global COVID-19 pandemic.

Abstract

With over 706 million confirmed cases and a terrible death toll over 6.9 million, the global repercussions of the COVID-19 pandemic have been astonishing. One noteworthy accomplishment stemming from the progress of modern science has been the rapid development and widespread distribution of COVID-19 vaccines. Extensive endeavors were undertaken to create an efficacious vaccine against the disease, reflecting the commitment and dedication of the scientific community.

The requirement for ultra-cold storage and varying permissible storage durations prior to vaccine administration has presented additional complexities, occasionally leading to errors during the vaccination rollout by deviations in the storage and handling procedures, such as inadequate temperature control or prolonged exposure of vaccines to ambient conditions prior to administration. The success of a vaccination campaign depends not only on the efficacy of the vaccine itself but also on the effective management of the cold supply chain. Therefore, there is an increasing need for vaccine platforms that offer improved stability, whether through novel formulations or the incorporation of new stabilizers. These advancements are crucial to ensure the viability and effectiveness of vaccines, addressing the challenges associated with storage and transportation in order to maximise the impact of vaccination efforts.

The aim of this research was to evaluate the influence of the macrocycle sCX[4] as a novel stabilizer in COVID-19 vaccine formulations on the conformational stability of the Pfizer-BioNTech and AstraZeneca vaccines. To achieve this objective, various fluorescence experiments were conducted using intrinsic tryptophan and external Nile red fluorescence to examine conformational changes of the virus or lipid carrier as the temperature increased. Additionally, dynamic light scattering, static light scattering, and transmission electron microscopy techniques were employed to gather additional insights into the particle size and morphology of the vaccine particles in the presence and absence of sCX[4]. The results

emphasise the considerable benefit of utilising sCX[4] as a stabiliser in the formulation of COVID-19 vaccines. The inclusion of sCX[4] demonstrated a notable advantage by effectively preserving the structural integrity of the virus and nanoparticles. This was evident through minimal alterations in light scattering intensity and maintained particle size distribution. These findings indicate that sCX[4] offers protection against aggregation and structural changes induced by elevated temperatures. To summarize, the application of advanced methodologies along with the incorporation of sCX[4] as a stabiliser presents encouraging outcomes in augmenting the thermal stability of COVID-19 vaccine formulations. Ongoing investigations will further enhance our comprehension of the ideal formulation and stability parameters for these vaccines, thereby enhancing their overall quality and effectiveness in combating the global challenges posed by COVID-19.

1.Introduction

The novel coronavirus disease 2019 (COVID-19) was initially recognized in December 2019 in Wuhan, China [1]. The rapid escalation of the COVID-19 outbreak within a few months resulted in its classification as a global pandemic in March 2020. The global impact of COVID-19 has resulted in over 706 million confirmed cases and a tragic death toll of 6.9 million people [2]. A notable achievement of contemporary scientific advancement has been the expeditious development and subsequent dissemination of vaccines against COVID-19. Numerous efforts were undertaken to develop an effective vaccine against COVID-19. The initial efficacious products were constructed utilising mRNA encoding the virus's Spike protein, encapsulated within a lipid envelope. The clinical trials of the Moderna and Biontech-Pfizer vaccines demonstrated favorable outcomes, leading to their widespread global implementation for the prevention of COVID-19 [3, 4]. Researchers have also achieved progress by utilising benign viruses as vectors to transmit information regarding virus proteins into human cells. Notably, Oxford University and AstraZeneca successfully employed a chimpanzee respiratory virus to elicit an immune response against the SARS-CoV-2 Spike protein, with promising outcomes [5]. Also, Johnson & Johnson has developed an effective vaccine that employs a similar strategy, utilising a modified version of human adenovirus 26 [6]. Several additional initiatives were also launched, building on previously established vaccine development concepts utilised for other diseases. Sinopharm (China) produced a vaccine following investigations involving inactivated SARS-CoV-2 virus [7]. Moreover, Novavax has produced a vaccine that utilises spike protein nanoparticles [8]. These products were promptly implemented for widespread vaccination campaigns.

Following the development of COVID-19 vaccines, vaccination efforts began in numerous countries. Pharmaceutical firms scaled up their production capacity, and mass vaccination sites

were established. However, the administration of these vaccines proved to be a complex undertaking, given the unique storage requirements and varying conditions for different vaccines. The need for deep-freezing and differing acceptable storage periods before administration to patients created additional challenges, occasionally resulting in errors during the vaccination process. The efficacy of a vaccination campaign hinges not only on the percentage of vaccine effectiveness but also on the proper management of the cold supply chain. A breakdown in this process can potentially render a highly effective vaccine useless, given the thermobile nature of vaccines [9-11]. Despite the storage of vaccines at low temperatures to preserve their stability and immunogenicity, their potency diminishes with time. Moreover, higher temperatures can accelerate this process of potency loss [12]. Inadequate storage conditions can impact vaccine stability, as evidenced by reductions in efficacy and changes to the safety profile [13-15], as measured by viral titers assays [16]. It is worth highlighting that low temperatures can also contribute to potency losses, such as the freezing-induced inactivation of aluminum-adjuvanted vaccines [17]. Moreover, The lipid nanoparticle (LNP) formulations can exhibit instability when subjected to freeze-thaw cycles, leading to potential alterations in their shape and encapsulation capacity under elevated temperatures [1].

The Pfizer-BioNTech COVID-19 vaccine contains a number of stabilisers, such as polythene glycol (PEG), an essential component of the lipid nanoparticle that encases the mRNA, and sucrose, which aids in maintaining the vaccine's stability while it is being stored. Also, several stabilisers are included in the AstraZeneca COVID-19 vaccine including L-histidine, sucrose, polysorbate 80, and ethylenediaminetetraacetic acid (EDTA). These stabilisers aid in preserving the adenoviral vector utilised in the vaccine under specified conditions. However, there is a need to improve the stability of these vaccines to overcome their limitations. Current

research efforts include identifying new strategies for improving vaccine stability, such as optimising the formulation and packaging of the vaccines [18-20].

The objective of this study was to assess the impact of macrocycle sCX[4] on the conformational stability lipid nanoparticle and virus vector of Pfizer-BioNTech and AstraZeneca COVID-19 vaccines, respectively. Thus, fluorescence experiments including tryptophan and Nile red fluorescence were conducted to observe conformational changes at temperature. Furthermore, dynamic light scattering, static light scattering, and transmission electron microscopy experiments were performed to provide more information on the particle size and morphology of the vaccines particles with and without the presences of the sCX[4].

2.Materials and Methods

2.1 Materials

All vaccines samples were leftover samples. sCX[4] was purchased from Sigma Aldrich (Massachusetts, USA). Copper TEM grids coated with carbon and Formvar were acquired from ProSciTech (Queensland, AUS). A Hellma quartz microcuvette (New York, USA) was utilised for all spectroscopic studies. Nile red and methanol were purchased from Sigma-Aldrich (Castle Hill, AUS). GraphPad Prism 9 was used to analyze the data.

2.2 Fluorescence emission spectroscopy

Fluorescence experiments were conducted using a Fluorolog 3 FL3-22 (Horiba Jobin Yvon, Japan). Vaccines samples were incubated for 30 minutes and tested with sCX[4] at 1.07 mM concentration. Samples were exposed to a temperature ramp from 25°C to 85°C with a 30-second equilibration time at each temperature point before measuring fluorescence. The tryptophan emission wavelength was measured from 290 to 450 nm at excitation of 280 nm

with 4 nm slits and 0.15 second integration time. The fluorescence emission spectrum of Nile red was recorded from 500 to 800 nm, with excitation at 490 nm with 4 nm slits and 0.15 second integration time. Each sample was evaluated using at least two separate aliquots (n = 2 or more), with necessary correction made for each spectrum by subtracting out the background signal. A 2 mM stock solution of Nile red was prepared in methanol and stored at 8°C. Then, 25 μL of this stock solution was added to 1000 μL water to prepare another diluted stock solution with 50 μM concentration. For each sample, 30 μL of the 50 μM Nile red stock solution was added to 70 μL of the sample (100 μL total).

2.3 Static and dynamic light scattering

The thermal stability of COVID-19 vaccines was evaluated using Uncle (Unchained Labs). Static light scattering signals were generated by laser-excited light at wavelengths of 266 nm during a temperature ramp. Changes in light scattering intensity were indicative of the aggregation process. Samples of 9 microliters were loaded in triplicate in Uni tubes (Unchained Labs, USA), and subjected to temperature ramping from 20°C to 80°C with an increment of 1°C per minute.

Uncle also provide dynamic light scattering information at the initial and final temperatures of the temperature ramp (20°C and 80°C). The measurements were performed in duplicate, and the standard errors were calculated using GraphPad Prism 9.

2.4Transition electron microscope TEM

JEOL JEM-1400 instrument (Tokyo, JPN), was utilised to acquire transmission electron microscopy (TEM) images of the COVID-19 vaccines. Distinct samples were arranged for each vaccine, where one sample was designated as a control, and the other samples were processed with or without 1.07 mM sCX[4] at various temperatures before loading onto TEM

grids. Negative staining was performed with a 2% w/v ammonium molybdate solution in water for a duration of one minute. Following this, the grids were blot-dried on filter paper and left to air-dry before imaging.

3. Results and discussion

3.1 Fluorescence emission spectroscopy

The conformational changes of AstraZeneca COVID-19 vaccines were assessed using the intrinsic tryptophan fluorescence (Fig.5-1). The intrinsic tryptophan fluorescence can be used as a method to study the stability of the adenovirus vector in the AstraZeneca vaccine. Tryptophan, an amino acid present in proteins, emits fluorescence when excited by ultraviolet light. The stability of a protein can be monitored by observing the changes in tryptophan emission spectrum, as unfolding or aggregation can cause changes in tryptophan environment, which is reflected on the fluorescent properties. By using this technique, the stability of the adenovirus vector in the AstraZeneca vaccine can be monitored and evaluated any potential changes or degradation over time.

Maximum fluorescence wavelength values for the AstraZeneca vaccination were seen between 25 and 55 ° C, however a sharp reduction was seen at 60 ° C (Fig.5-1). At reaching temperatures over 60° c, the maximum fluorescence wavelength showed no discernible shifts. The maximum fluorescence wavelength was found to decrease significantly from 25 to 60 ° C, from around 323 to 310 nm. This blue shift in wavelength is consistent with earlier studies suggesting structural changes leading to protein unfolding and the development of aggregates [21-23].

Several macrocycles, including sCX[4], have been tested for their ability to 145tabilizi the influenza virus at varying doses in earlier studies. Of them, sCX[4] at a concentration of 1.07 mM showed very promising results. The macrocycle sCX[4], which showed the most promising results among the evaluated choices, was used at concentrations of 0.53 mM, 1.07 mM, and 2.1 mM to preserve vaccination samples for future tests. The purpose of this method

was to establish the sCX[4] concentration necessary to provide the desired 146tabilizing effects on the examined vaccinations. The fluorescence emission spectra of the sCX[4]-treated samples were stable throughout a broad temperature range, whereas those of the control samples shifted to the blue (Fig.5-1). In contrast to the concentrations of 1.07 mM and 2.1 mM sCX[4], the application of 0.53 mM sCX[4] resulted in a noticeable blue shift in the wavelength at the maximum temperature of 85°C, suggesting a much less stability impact. Based on the data, it seems that the effects of sCX[4] doses of 1.07 mM and 2.1 mM are statistically equivalent. Hence, the 1.07 mM concentration may be seen as a more desirable option since it permits the use of a lesser quantity of stabiliser in the vaccine formulation while yet offering similarly beneficial stability. The finding suggest that sCX[4] binding could stabilise the virus structure, thereby preventing conformational alterations. Another potential mechanism is that sCX[4] interacts with tryptophan residues in the protein, protecting them from the effects of temperature increase and preserving the peak wavelength. The control sample lacked stabilizing factors, leading to conformational changes, as evidenced by the blue shift in the fluorescence emission spectrum.

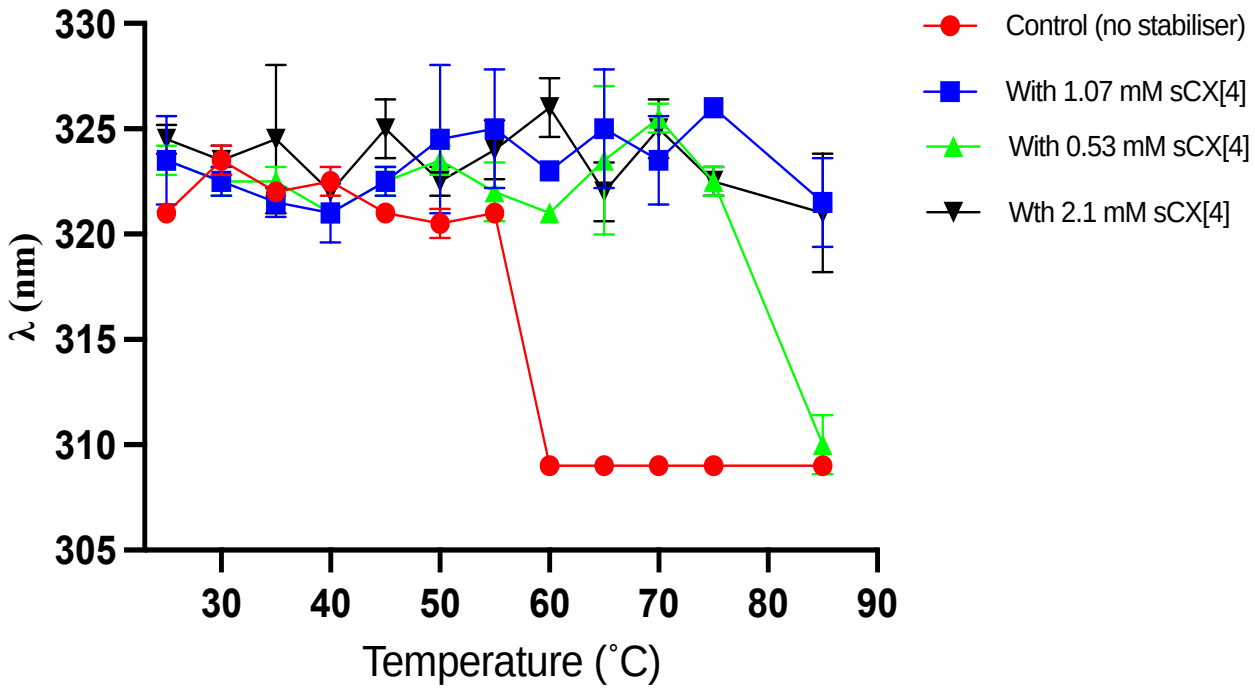

Figure 5-1: Changing of the wavelength at maximum fluorescence emission spectra of Tryptophan in AstraZeneca vaccine samples with/without sCX[4].

The stability assessment of Pfizer vaccine samples was carried out using Nile red dye fluorescence instead of tryptophan due to its absence in the vaccine. Extrinsic fluorescence was employed in this case as opposed to intrinsic fluorescence to evaluate the vaccine stability (Fig.5-2).

Nile red (NR), a hydrophobic fluorescent dye, is capable of binding to a variety of hydrophobic domains found in biological samples, such as lipids [24-30], proteins aggregates [31-34], surfactant micelles [35-37], the hydrophobic regions of certain proteins [24, 32]. As a result, Nile red fluorescence emission has numerous biological applications. Alterations in polarity and hydrophobicity of the microenvironment can modify the intensity and wavelength of the fluorescence signal [25, 29, 35]. The presence of lipids and other hydrophobic particles, such as protein aggregates, provides more hydrophobic environments for NR to bind to, which results in increased fluorescence.

Here, the study conducted on the stability of the Pfizer vaccine using NR dye fluorescence showed a sharp decrease in the NR intensity with an increase in temperature from 25˚C to 85˚C (Fig.5-2). At 85 ° C, the intensity was shown to be around 40% of what it was at 25 °. The rupture of the vaccine particles' lipid membrane may account for this finding. A reduction in fluorescence intensity may arise if the NR dye has a less hydrophobic environment to adhere to.

Vaccine samples incubated with the stabilisers exhibited no dramatic decline in intensity with rising temperature, in contrast to the control samples. There was no noticeable change between 1.07 mM and 2.1 mM sCX[4]-treated samples, mirroring findings with the AstraZeneca vaccine. Pfizer vaccination samples exposed with lower concentrations of sCX[4] (0.53 mM) showed a smaller reduction in NR intensity compared to those incubated with higher concentrations (1.07 mM and 2.1 mM). These results suggest that sCX[4] at a concentration of 1.07 mM may be the best option for use as a stabiliser in the vaccine formulation.

The stabilising agents attach to the lipid membrane of the lipid particles and prevent their disruption, which may explain the observed stability. As a result, the NR fluorescence intensity remained more stable over a wider temperature range. Overall, the presence of the stabiliser seems to have an impact on the stability of the vaccine, allowing it to maintain its fluorescence intensity over a wide temperature range.

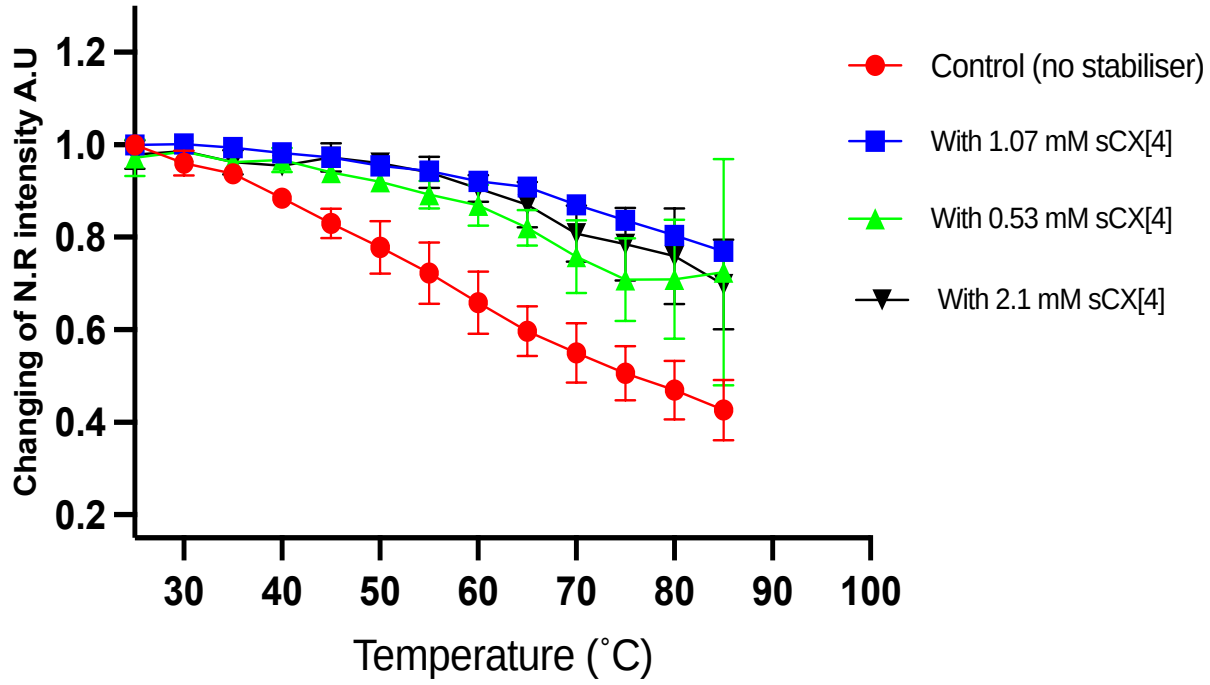

Fig. 5-2. The change of fluorescence intensity of Nile red dye as a function of Temperature after incubation of Pfizer vaccine samples with/without 1.07 mM sCX[4].

3.2 Static and dynamic light scattering

Protein thermal stability is often evaluated with the use of the Optim equipment by Unchained Labs. We used Uncle to track 266 nm laser-induced light scattering signals as a function of temperature in this investigation. The changes in light scattering intensity were indicative of the protein aggregation process. Here, vaccination samples from AstraZeneca were heated from 20 ° C to 80 ° C at a rate of 1 degree Celsius per minute (Fig.5-3). Without the 149tabilizer, the AstraZeneca vaccination sample showed clear signs of aggregation, as measured by an increase in light scattering intensity at 60°C. This indicates that the aggregation process has started, and it grew dramatically when the temperature was raised during the thermal ramp, suggesting that precipitation of aggregated material has commenced (Fig.5-3).

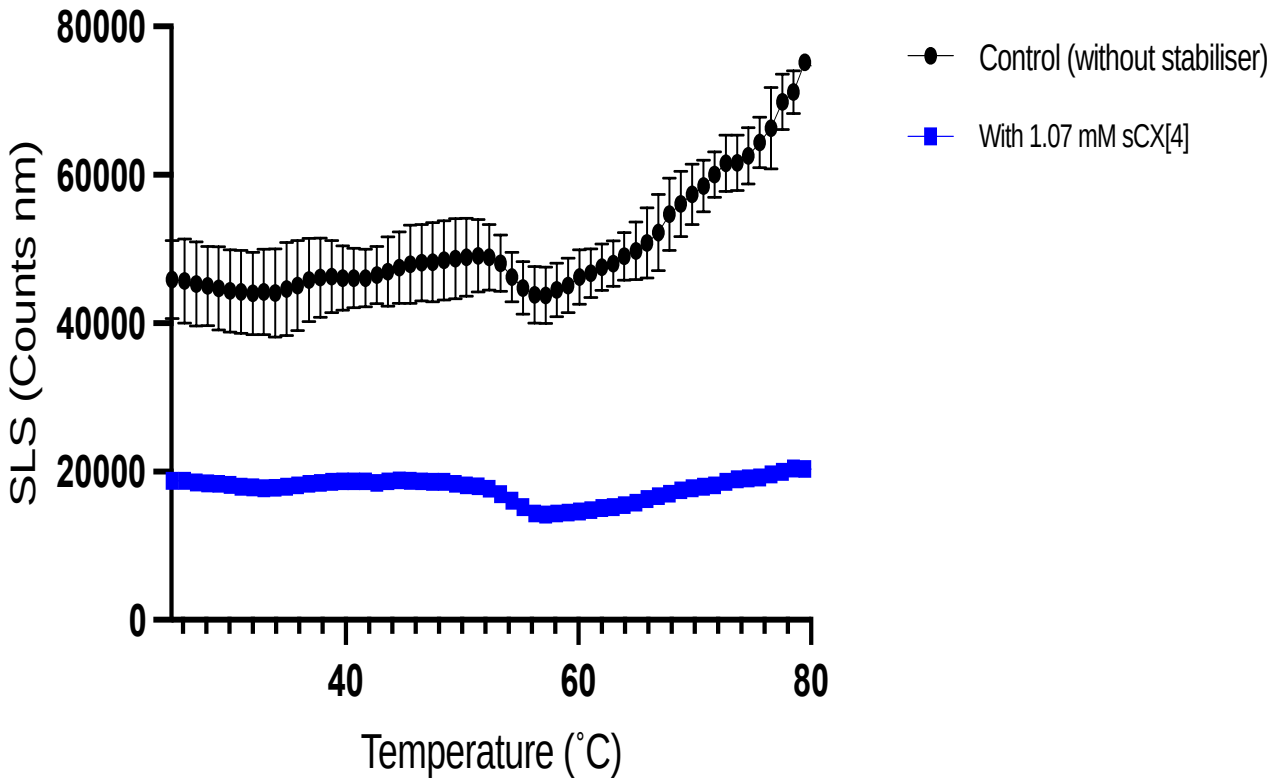

Fig. 5-3. Aggregation curves for AstraZeneca vaccine samples with/without 1.07 mM sCX[4] as measured by scattering intensity at 266 nm over a thermal ramp.

Proteins in the AstraZeneca vaccination sample likely aggregated and precipitated due to the viral vector particles' destabilisation, which exposed hydrophobic areas. High temperatures may also promote aggregation by denaturing the proteins and disrupting the lipid membrane. After the thermal ramp, the AstraZeneca vaccination samples stabilised with 1.07 mM sCX[4] showed much less dispersed light intensity than those without stabilisation, with the rise beginning about 60 degrees Celsius (Fig.5-3). As a result, sCX[4] may be able to stabilise the AstraZeneca vaccination and inhibit the beginning of aggregation when exposed to high temperatures. The stabiliser likely prevents the exposure of hydrophobic residues in the vaccine proteins to aqueous solvents, thereby maintaining their structural integrity and stability. Additionally, sCX[4] may help preventing the onset of aggregation and subsequent

precipitation of material by stabilising the folded structure of the proteins in the vaccine. The static light scattering at 266 nm was not informative for the Pfizer vaccine samples, possibly due to the absence of proteins in the vaccine formulation. Additionally, other factors such as the presence of lipids, salts, or other components in the vaccine formulation could also influence the light scattering signal.

The Uncle instrument offers the capability to obtain quantitative sizing and measurements at the start and end of a thermal ramp experiment. This allows for additional information to be gathered without the need for sacrificing more sample. Moreover, this can be achieved with only a slight increase in the overall experimental time, thereby enhancing the data set with valuable sizing and characterization data.

At the initial stage of the thermal ramp experiment for the Pfizer vaccine sample (at 20°C), we observed the presence of aggregation for larger particles exceeding 200 nm. However, there was also a distinct peak corresponding to a population of particles around 100 nm, which represents the vaccine particles themselves (Fig.5-4). At the final stage of the thermal ramp experiment (80°C) for the Pfizer vaccine samples, we observed the disappearance of the peak representing individual vaccine particles around 100 nm. Instead, a distinct peak corresponding to a population of small particles around 20 nm was observed. This indicates a significant disruption and potential breakdown of the vaccine particles at higher temperatures. The observed changes in particle size distribution suggest that the temperature-induced effects may lead to the disintegration or decomposition of the vaccine particles, resulting in the formation of smaller fragments. Additionally, factors such as the absence of stabilising proteins in the Pfizer vaccine formulation and the susceptibility of lipid-based carriers to temperature variations could contribute to the observed disruption of the vaccine particles.

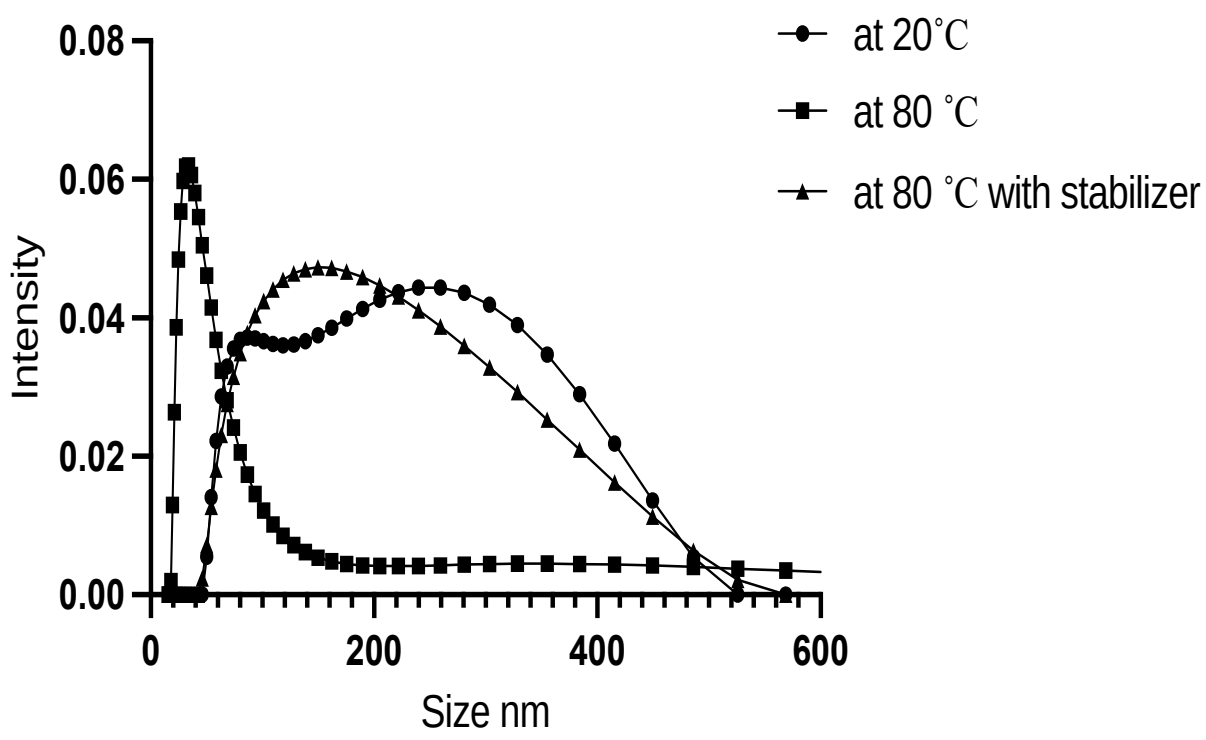

Fig. 5-4. The hydrodynamic size of Pfizer vaccine samples strains using Uncle. A control sample at the beginning of the thermal ramp (20 ˚C) was compared to samples at the end thermal ramp (80 ˚C) with and without sCX[4].

In contrast, when the Pfizer vaccine samples were incubated with sCX[4] at 80°C (at the end of the thermal ramp), the size distribution remained similar to that observed at the beginning of the thermal ramp at 80°C. This indicates that the presence of sCX[4] may have played a role in preserving the structural integrity of the vaccine particles under elevated temperatures. The stabilising effects of sCX[4] could have helped mitigate the temperature-induced disruptions and maintain the size distribution of the vaccine particles. Additionally, the interaction between sCX[4] and the components of the vaccine formulation, such as lipids or other stabilising agents, may have contributed to the protective effect observed.

At the onset of the thermal ramp, AstraZeneca vaccine samples at 20°C displayed a prominent peak in the size distribution corresponding to population particles around 100 nm, indicative of the presence of intact vaccine particles (Fig.5-5).

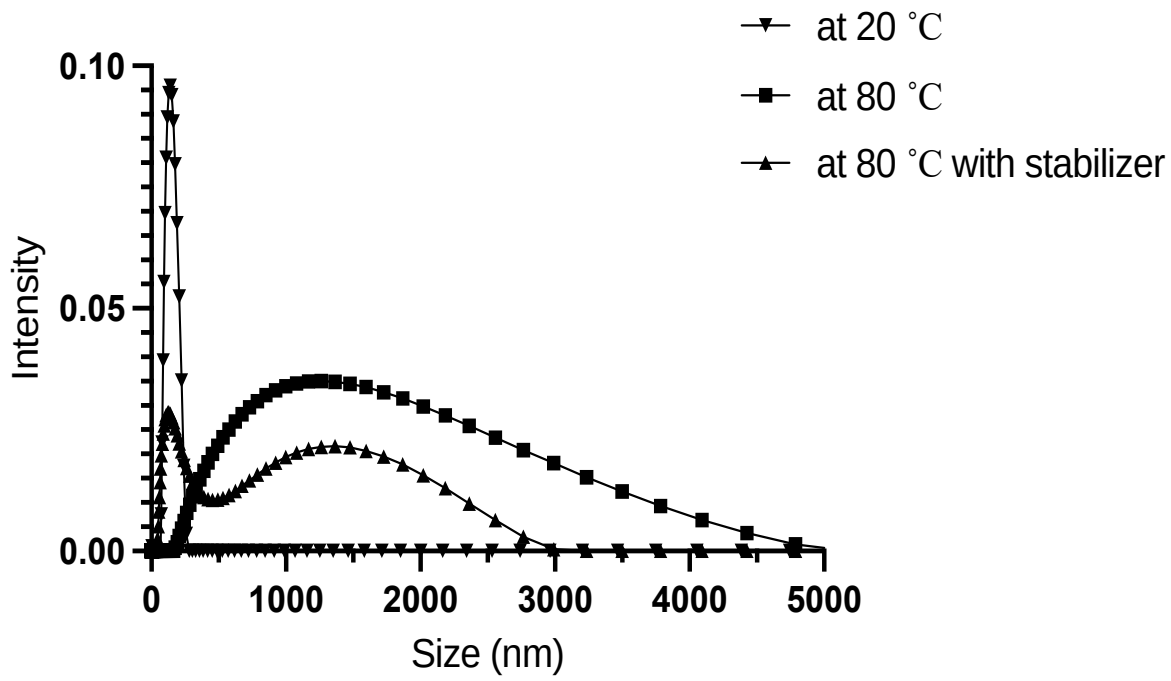

Fig. 5-5. The hydrodynamic size of AstraZeneca vaccine samples strains using Uncle. A control sample at the beginning of the thermal ramp (20 ˚C) was compared to samples at the end thermal ramp (80 ˚C) with and without sCX[4].

At the end of the thermal ramp at 80°C, the AstraZeneca vaccine samples exhibited the absence of the peak representing individual vaccine particles around 100 nm, indicating a loss of structural integrity. Instead, a new peak emerged in the size distribution corresponding to larger particles around 1000 nm, indicative of aggregation. This aggregation was also observed by static light scattering using Uncle and reinforces the notion that the temperature-induced stress resulted in the aggregation of the vaccine particles. Possible factors contributing to this aggregation include the 153estabilizing effects of elevated temperature on the viral vector and the disruption of stabilizing interactions within the vaccine formulation.

In contrast to the AstraZeneca vaccine samples without sCX[4] at 80°C, the samples treated with sCX[4] exhibited a notable preservation of the peak representing individual vaccine particles around 100 nm (Fig.5-5). Although some degree of aggregation was still observed,

indicating a moderate impact of the elevated temperature, the presence of the peak suggests a stabilising effect conferred by sCX[4]. This indicates that the addition of sCX[4] may contribute to maintaining the structural integrity and stability of the vaccine particles under thermal stress. The stabilisation effect could be attributed to the ability of sCX[4] to interact with vaccine components, potentially preventing or mitigating the aggregation process.

3.3 Transition electron microscope TEM

TEM permits direct visualisation of vaccine particle size, shape, and surface morphology. This can provide valuable information regarding the composition and structure of vaccine particle, which can enlighten their stability. Electron microscopy was utilised as a supplementary approach to fluorescence and light scattering (dynamic and static) spectroscopy to obtain more insightful information about the effect of sCX[4] on the stability of COVID-19 vaccines. Electron microscopy's superior image quality allows for more detailed studies of vaccine structural diversity. Additionally, it provides complementary data to further explicate the influence of the stabiliser on the vaccines when exposed to stressful conditions, such as heat.

Here, TEM was used to look at what happened to the structure of COVID-19 vaccinations after they were incubated without a stabiliser at three different temperatures (at 30, 40, and 60 ° C). The results showed that the vaccine samples exhibited structural changes under stress conditions. Furthermore, the extent of structural changes was greater at higher temperatures (60˚C) compared to lower temperatures (30˚C) for both vaccine samples (Fig.5-6). Temperature-induced denaturation and aggregation of surface proteins contained in vaccine formulations might explain the structural alterations observed in COVID-19 vaccines with increasing temperature. Pfizer and AstraZeneca vaccines both include lipids or proteins that are prone to denaturation and aggregation under stress conditions. The lipid nanoparticles in the Pfizer vaccine, for example, are made of ionizable cationic lipids, polythene glycol (PEG)

lipids, and cholesterol, all of which are known to be temperature sensitive. Similarly, the AstraZeneca vaccine contains adenoviral vectors covered with viral proteins and lipids, which are prone to denaturation and aggregation at high temperatures.

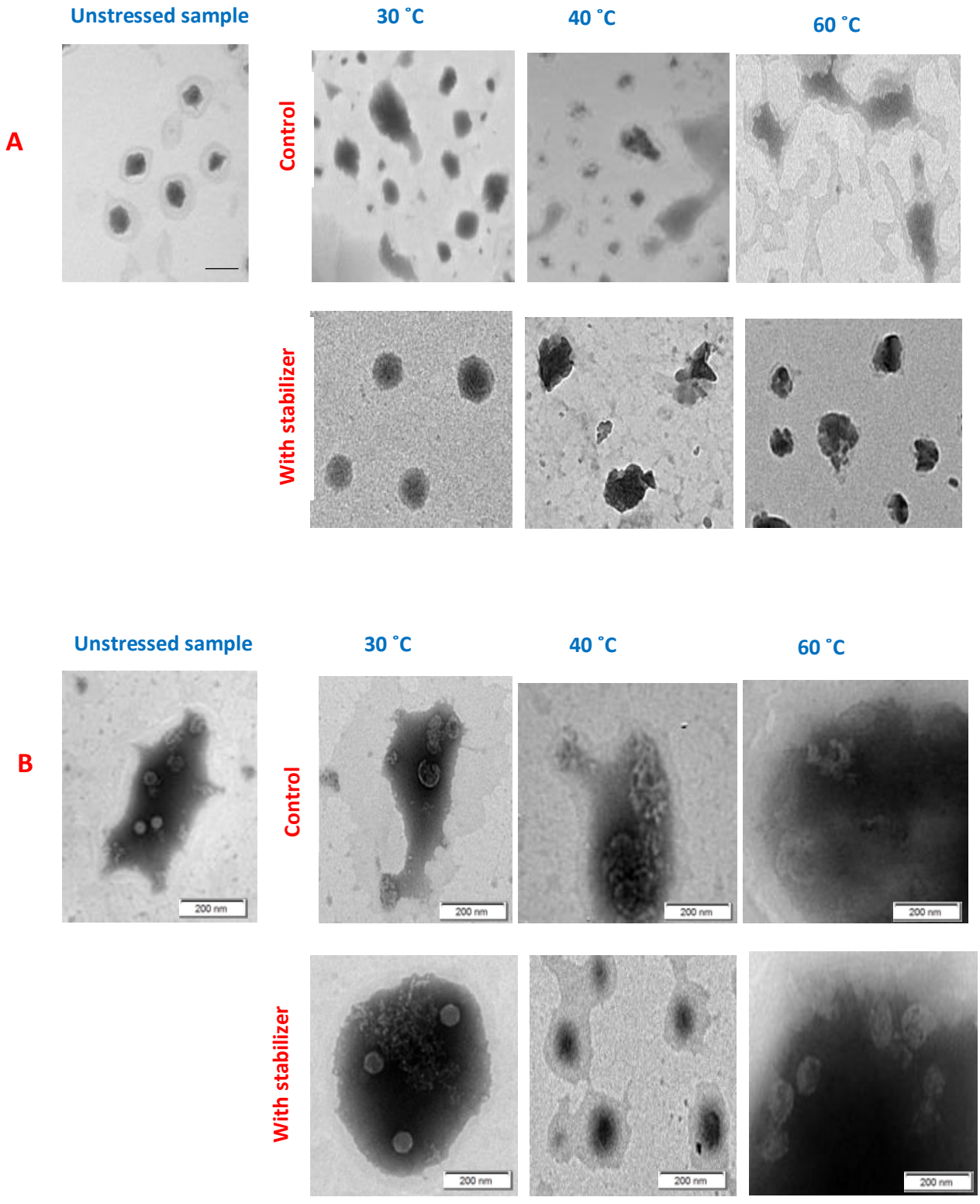

Fig. 5-6. Electron micrographs of negatively stained COVID-19 vaccines. A Pfizer vaccine, B AstraZeneca vaccine. Each vaccine has unstressed sample, control sample and sample with 1.07 mM sCX[4] at 30 ˚C, 40 ˚C , and 60 ˚C. The scale bar (—) represent 200 nm.

We found that the vaccination samples had some structural integrity even after being incubated at the same temperatures with 1.07 mM of sCX[4], which may be due to the compound's stabilising actions. Nevertheless, the stability of the vaccinations was shown to be temperature dependent, with a greater impact being seen at lower temperatures. The stabilising function of sCX[4] in Pfizer vaccine may be explained by its interaction with the lipid membrane of the vaccine particles, which aids in maintaining their structural integrity under stress. As the AstraZeneca vaccine is a viral vector vaccine, it naturally includes viral proteins; the stabilising effect of sCX[4] might be due to its capacity to bind with these proteins, hence decreasing the chance of conformational changes and protein unfolding under stress. The sCX[4] may potentially stabilise the vaccination by interacting with the lipid membrane of the vaccine particles.

4. Conclusion

This in-depth analysis sheds light on the thermal stability and aggregation behaviour of COVID-19 vaccine formulations, which is crucial information for developing effective vaccines. We used fluorescence spectroscopy, dynamic and static light scattering, and electron microscopy to determine how the stabiliser sCX[4] affected the vaccination preparations.

The results show how beneficial it is to include sCX[4] as a stabiliser in the COVID-19 vaccines. The vaccines' structural integrity was well retained by sCX[4], as shown by little variation in light scattering intensity and unaltered particle size distribution. This provides support for the idea that sCX[4] may shield against the aggregation and structural disturbances that high temperatures produce.

Yet, further research is needed to determine whether or not the stabiliser has a negative effect on the vaccine's effectiveness. To guarantee that sCX[4] does not damage the vaccine's immunogenicity or its ability to protect against COVID-19, it must be tested in settings where sCX[4] is present.

In conclusion, the findings from including sCX[4] as a stabiliser and using cutting-edge procedures show promise for improving the thermal stability of COVID-19 vaccine formulations. Improved vaccine quality and effectiveness in the worldwide battle against COVID-19 will result from further research into the appropriate formulation and stability conditions for these vaccines.

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Chapter 6

Conclusion

Three major facets of vaccine development and stability have been investigated in these in-depth investigations. These studies not only add to the body of knowledge on vaccine production, but also have important implications for the creation of influenza and COVID-19 vaccines that are both safe and stable.

The first study looked for ways to get around Triton X-100's shortcomings while making inactivated flu vaccines. We have found that Brij-56 is a potential alternative to Triton X-100 for accomplishing viral splitting in the manufacturing of inactivated split-virus influenza vaccines. Brij-56 was shown to effectively interact with the influenza virus, resulting in viral splitting in a complete examination that included fluorescence, light scattering, and transmission electron microscopy. These results support Brij-56's viability as a surfactant, suggesting it may be used to improve the safety of influenza vaccinations and circumvent the drawbacks seen when using Triton X-100.

In the second study, we looked at how sCX[4] affected inactivated influenza virus as a whole. We have determined that sCX[4] has promise as a stabiliser candidate for influenza vaccinations by our analysis of conformational stability and decrease in aggregation formation. By showing that sCX[4] improves the virus' conformational stability and decreases aggregate formation, the work sheds light on the usefulness of this compound in maintaining the structural integrity of influenza vaccinations. To learn more about the synergistic effects and general applicability of vaccine formulations, future studies should include other excipients typically used in such preparations.

In the third study, we looked at how well COVID-19 vaccines reacted to heat and how they clumped together. Using the use of cutting-edge methods including fluorescence spectroscopy, light scattering, and electron microscopy, we were able to see the benefits of the stabiliser sCX[4] in preserving the structural integrity of the vaccinations. The results of this study provide important information for improving the stability of COVID-19 vaccines by showing

that the inclusion of sCX[4] protects against aggregation and structural disturbances induced by increased temperatures. Nevertheless, further research is needed to determine the effect of sCX[4] on vaccination effectiveness and immunogenicity, so that the intended immune response against COVID-19 is not compromised.

The results of these analyses taken as a whole highlight the relevance of these research to the creation and maintenance of vaccines. A major shortcoming of inactivated influenza vaccines has been addressed by the discovery of Brij-56 as a suitable replacement for Triton X-100, and new opportunities for enhancing vaccine formulations have been unlocked by the discovery of sCX[4] as a stabiliser for both influenza and COVID-19 vaccines. These results add to the growing body of evidence supporting the need to improve vaccines' stability and safety.

There are several holes in the discipline that need to be filled by future study. As a first step, a thorough knowledge of the combined effectiveness of sCX[4] and other excipients routinely used in vaccine formulations may be gained by investigating their synergistic effects. The adjuvant characteristics of macrocycles and surfactants have been observed, suggesting a possible enhancement in vaccine effectiveness. Nevertheless, more research is necessary to ascertain their compatibility with the specific immune response being targeted and the components of the influenza vaccine. This necessitates doing extensive investigation on the immunogenicity and overall protective effectiveness of vaccinations that include sCX[4] or Brij 56.

In addition, researchers should keep working to perfect COVID-19 vaccine stability and formulation. Studying the influence of storage and transportation circumstances on vaccine durability, for example, is part of this process, as does evaluating the vaccine's efficacy against new variations. Nanoparticle-based delivery methods and innovative adjuvants are two examples of cutting-edge technology that might be used to improve vaccination efficacy and stability.

In conclusion, the results of these studies provide important new perspectives on how to improve and preserve vaccines. Brij-56 and sCX[4] are two examples of alternative surfactants and stabilisers that show promise for addressing current issues and enhancing the safety and stability of influenza and COVID-19 vaccines. These results are important for the development of vaccine production and might have far-reaching effects on public health.