A Literature Review on the Potential Use of Nanotechnology to Deliver Prophylactic Vaccines for Influenza Viruses

By

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

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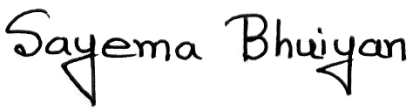

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Approval

The thesis/project titled “Potential use of Nanotechnology to deliver Prophylactic Vaccines for Influenza Viruses” submitted by Sayema Bhuiyan (16346016) of Summer, 2016 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

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Ethics Statement

The study does not involve any kind of animal trial and human trial.

Abstract

The influenza virus is the cause of the life-threatening influenza disease, which kills thousands of people each year. Vaccination is the most effective approach to avoid it. To prevent the influenza virus, many types of conventional vaccines have been employed, each of which is designed for a specific strain. However, there is still a risk of an influenza outbreak due to antigenic drift and shift. Nanotechnology platform is used in vaccine development to overcome the drawbacks of conventional vaccinations, which employ nanoparticles with a size less than 100nm. Moreover, targeted delivery, long-term release and therefore long-lasting effectiveness, and single-dose vaccination are all advantages of nanoparticle vaccines over conventional vaccines. Some influenza nanovaccine that have been on the market for a few years and are effective against some strains of the influenza virus. Researchers have been working to develop a universal influenza nanovaccine that would give protection against all influenza virus strains. This review aims to look at how nanotechnology could be used to develop and manufacture prophylactic influenza vaccines, as well as several prophylactic nanovaccine trials that have been successful and are currently in various phases of clinical stages. Furthermore, this review will concentrate on all of the possibilities that future researchers would have with various forms of nanocarriers in the combat against influenza virus.

Keywords: Influenza; Nanotechnology; Nanovaccine; Nanocarriers; Prophylactic vaccination.

Dedication

I want to dedicate my project to my parents who have always been there to support.

Acknowledgement

At first, I would like to convey my gratefulness to Almighty Allah for providing me with the strength and patience required to accomplish my project work. I wouldn't be able to finish my project work without Allah's guidance.

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List of Acronyms

IAV	Influenza A virus
IBV	Influenza B virus
HA	Hemagglutinin
NA	Neuraminidase
NPs	Nanoparticles
VLPs	Virus-like particles
NP	Nucleoprotein
M1/M2	Matrix protein 1 and 2
APCs	Antigen-presenting cells
MHC	Major histocompatibility complex
DCs	Dendritic cells
RNP	Ribonucleoprotein
RdRp	RNA-dependent RNA polymerase
NALT	Nasal-associated lymphoid tissues
IL	Interleukin
M-cell	Microfold cell;
pIgR	Polymeric immunoglobulin receptor
sIgA	Secretory IgA

rHF	Assembled human heavy chain ferritin cage
LAIV	Live attenuated influenza vaccine
CTL	cytotoxic T-lymphocyte
M2e	Ectodomain of matrix protein 2
Th cells	T-helper cells

Chapter 1

Introduction

1.1 Influenza virus-cause of a terrifying epidemic for human civilization

Influenza is kind of a virus that produces a moderate to serious illness that can result in death (Raffael Nachbagauer & Palese, 2020). The influenza virus belongs to the Orthomyxoviridae family of viruses (Applications, Wieczorek, Szutkowska, & Kierzek, 2020). The Influenza viruses, which are segmented and negative-stranded (Raffael Nachbagauer & Palese, 2020), can cause influenza that is an infectious respiratory disease (Rungrojcharoenkit et al., 2020).

There are four influenza viruses that can be distinguished: A, B, C (known to be human-infectious), and D (unconfirmed to be human-threatening). The influenza A virus (IAV) and influenza B virus (IBV) are two of these viruses that have the potential to cause annual epidemics, also known as seasonal flu (Applications et al., 2020), and are responsible for the majority of human illness (Raffael Nachbagauer & Palese, 2020). Along with seasonal flu, influenza viruses caused pandemics at several times (Krammer & Palese, 2015). However, only influenza A viruses have been known to cause pandemic outbreaks due to viruses circulating in animal reservoirs, and as influenza B viruses are believed to depend on only the humans as their primary host (Raffael Nachbagauer & Palese, 2020). Conversely, the influenza C and D viruses are not considered to be dangerous for health. The influenza C virus causes a mild illness in humans, whereas the influenza D virus is incapable of infecting humans (Francis, King, & Kelvin, 2019).

Hemagglutinin (HA) and neuraminidase (NA) are the two main surface glycoproteins that coat the influenza virus. For cell entry, HA mediates both receptor (glycan) binding and membrane

fusion, while NA serves as a receptor-destroying enzyme during virus release. Viruses of influenza A are divided into 16 HA (H1–H16) and 9 NA (N1–N9) subtypes (Gopinath & Kumar, 2013). H1N1 and H3N2, the subtypes of IAVs, are extremely common in humans, occurring in the population at all times and causing seasonal outbreaks (McAuley, Gilbertson, Trifkovic, Brown, & McKimm-Breschkin, 2019). Influenza B viruses, on the other hand, are classified not by subtypes but by two lineages (B/Victoria lineage and B/Yamagata lineage), each of which contains antigenically changing strains (Kanegae et al., 1990). Furthermore, both influenza A and B viruses have HA, NA, and M2 proteins on their surfaces, as well as eight negative-sense single-stranded RNA segments (Francis et al., 2019).

Antigenic shift and antigenic drift, which are responsible for repeating infection, are the two primary mechanisms of mutation change in influenza viruses (Francis et al., 2019). Antigenic drift is the process through which the genomes of circulating influenza viruses change over time, allowing the virus to trigger yearly epidemics. Antigenic drift occurs when changes in the haemagglutinin and neuraminidase genes occur, altering the antigenicity of these proteins to the point that antibodies specific for previously circulating strains no longer neutralize the ‘drifted' strains (Iwasaki & Pillai, 2014). Conversely, antigenic shift is the formation of a new virus subtype with mixed HA and NA from different subtypes (J. Chen & Deng, 2009). Novel pandemic influenza A viruses differ from season influenza A viruses in terms of the composition of their major surface antigens (Herzog et al., 2009) as seasonal influenza epidemics are caused by antigenic drift, whereas pandemic influenza virus outbreaks are caused by shift (Francis et al., 2019).

Human influenza virus infections have a significant public health and economic impact all over the world (R. Nachbagauer & Krammer, 2017). According to the World Health Organization

(WHO), annual influenza epidemics cause 3–5 million instances of severe illness and 290,000 to 650,000 deaths globally each year (Raffael Nachbagauer & Palese, 2020). Annual influenza epidemics, according to the World Health Organization (WHO), result in 3–5 million severe illness cases and 290,000–650,000 deaths globally each year (Raffael Nachbagauer & Palese, 2020). The 1918 influenza virus pandemic, which was caused by the H1N1 virus, took the lives of nearly 40 million people. Since then, pandemics have been caused by H2N2, H3N2, and H1N1 in 1957, 1968, and 2009 respectively (Krammer & Palese, 2015).

1.2 Different ways to prevent the onset and spread of Influenza

Influenza vaccine and antiviral medicines are used to control influenza infections in humans across the world (Rungrojcharoenkit et al., 2020).

Vaccination is the most effective way to reduce the severity of influenza virus infections (Mcmillan et al., 2021). The global population is likely to lack immunity to the pandemic virus strain and will require two vaccination doses to achieve protective protection (Rungrojcharoenkit et al., 2020). Current influenza virus vaccinations are effective and provide the best protection against both seasonal and pandemic influenza virus infections (Bhardwaj, Bhatia, Sharma, Ahamad, & Banerjee, 2020).

Even if vaccinations remain the most effective technique of avoiding influenza infection, antivirals have an essential role in the treatment of influenza, particularly for hospitalized and critically sick infected patients. Antivirals will also play a key role in the treatment and prevention of influenza infections in the event of a pandemic, because particular vaccinations will take months to develop. M2 ion channel blockers (rimantadine, amantadine (J. Chen & Deng, 2009)) and neuraminidase inhibitors (oseltamivir, zanamivir, peramivir (J. Chen & Deng, 2009)) are two types of influenza antivirals that have acquired universal approval across

the world (NAIs). Along with that, a drug from the polymerase inhibitor class of antivirals has also been granted restricted approval in Japan (Farrukee & Hurt, 2017). Antiviral effectiveness is being hampered by growing resistance to both M2 and NA inhibitors (Rungrojcharoenkit et al., 2020).

1.3 Existing prophylactic vaccines to weaken the spread of Influenza viruses

Influenza vaccinations are widely regarded as the most effective means of preventing pandemic influenza (Rungrojcharoenkit et al., 2020). The influenza hemagglutinin (HA) protein has been the primary target for vaccines because it is large and easily accessible on the viral surface, is required for viral binding and infection of infected cells, and is the primary target to induce immune responses (Mcmillan et al., 2021). However, due to the high mistake rate of the viral polymerase and selection from immunological pressure of circulating anti-bodies, the major antigen of the influenza virus, haemagglutinin (HA), has a very high plasticity and changes continually (Bhardwaj et al., 2020). Traditional influenza vaccines, which are manufactured in the millions every year, are made by inactivating and dividing influenza viruses cultured in hen embryonated eggs (Mcmillan et al., 2021).

1.4 Vaccination

Vaccination has been one of the most significant public health initiatives in the control of many diseases and the promotion of good health since Edward Jenner invented the first vaccine in 1796 (Gheibi Hayat & Darroudi, 2019). A vaccine is a preparation of a weakened or killed pathogen, such as a bacterium or virus, or a portion of the pathogen's structure, that stimulates antibody development or cellular immunity against the pathogen after administration but is unable to trigger any serious infection (Sekhon, Sekhon, & Saluja, 2014). Vaccines are made up of a biochemical agent that acts like a disease-causing microorganism which boosts

immunity to that disease (Vijayan, Mohapatra, Uthaman, & Park, 2019). A suspending fluid (sterile water, saline, or protein-containing fluids), preservatives and stabilizers (albumin, phenols, and glycine, for example), and adjuvants or enhancers that further increase the vaccine's efficacy are widely used chemicals in the development of vaccines (Sekhon, Sekhon, & Saluja, 2014). Vaccines work by activating the immune system to launch an immune response against particular targets, such as a cancer cells and microbial agents (Gill, 2013).

1.5 Downsides of Traditional Vaccination

Researchers have been trying to discover a treatment for most frequent pandemic diseases by immunotherapy and vaccination, which has effectively eradicated a number of common diseases. However, off-target responses, a lack of long-term resistance against a range of pathogenic strains, and allergy prevent it from being used to prevent or cure other globally prevalent infections like influenza (Bhardwaj, Bhatia, Sharma, Ahamad, & Banerjee, 2020). Prophylactic vaccinations use live-attenuated pathogens (Bhardwaj et al., 2020), which have little need for protection (Gheibi Hayat & Darroudi, 2019), or inactivated pathogens (Bhardwaj et al., 2020), toxoids, and recombinant pathogens, which may not properly trigger the immune system and require booster doses or adjuvants (Gheibi Hayat & Darroudi, 2019), but the risk of co-administered adjuvants and pathogen reversion is a matter of concern for their safety and effectiveness (Bhardwaj et al., 2020). For instance, while influenza vaccinations are approved, their efficiency in older persons is greatly reduced due to the innate and adaptive immune systems' lowered capacities (Ross, Senapati, Alley, & Darling, 2019).

Additionally, some new vaccine modalities, such as subunit vaccines and DNA vaccines that encode antigenic pathogenic proteins, have recently been investigated (Kim, Yeon, Shon, & Kim, 2014). Even as subunit and DNA vaccines have a higher safety profile than traditional

vaccines, they have a lower immunogenicity (Kim, Yeon, Shon, & Kim, 2014) and therefore require adjuvants to induce sufficient immunity (Sekhon et al., 2014). Moreover, vaccines that do not need a cold chain are still in demand (Gheibi Hayat & Darroudi, 2019).

Consequently, considering the success of traditional vaccines, significant changes are expected due to questions about the vaccines' poor immunogenicity, intrinsic instability in vivo, toxicity, and the need for several administrations (Kim et al., 2014). Hence, there is always a constant demand for new and improved vaccines (Sekhon et al., 2014).

1.6 Nanotechnology-Mitigation of the drawbacks associated with traditional vaccines

To overcome problems related to conventional vaccines, nanotechnology platforms have recently been incorporated into vaccine development (Kim et al., 2014). Nanotechnology is an advanced science that deals with the development and manufacturing of elements and hardware at the atomic scale between 0.1 and 100 nanometers, and nanoparticles are particles with a diameter of less than 100 nanometers (Sekhon et al., 2014).

The ability to achieve size-dependent transmission to lymphoid organs, the formation of antigenic depots that improve the immunogenicity of antigens but which result in long-term immune response fatigue, and the ability to change the surface of the particles with antigenic or adjuvant moieties to imitate pathogens, and finally the absorption of nanoparticles by APCs improves the cross-presentation of antigens on MHC I are all the key benefits gained from the use of nanotechnology in immunotherapy (Fontana, Figueiredo, & Santos, 2019). Moreover, due to their limited size and modifiability, nanoparticles demonstrate considerable potential in cancer treatment by selectively obtaining entry to tumors (Gill, 2013).

Different nanovehicles, like liposomes, nanoparticles, microparticles, dendrimers, and micelles, etc., are gifts in nanotechnology and are well known for their ability to protect the encapsulated antigen (Sekhon et al., 2014). Nanoparticles are formulated from a variety of substances and fabricated to carry a wide range of substances in a controlled and targeted manner (Gill, 2013).

1.7 Nanovacccines-An advanced approach with improved immunogenicity

The application of nanotechnology in the field of vaccinology came into play in order to press forward with enhancing immunogenicity, resulting in the name nanovaccinology. (Yadav et al., 2018). Nanoparticle vaccines (nanovaccines) activate the innate immune system and boost adaptive immunity while lowering toxicity (Luo, Samandi, Wang, Chen, & Gao, 2017), and have been studied by combining natural or synthetic nanomaterials with pathogen-specific antigens to induce a controlled immune response (Bhardwaj et al., 2020).

Nanovaccines outperform traditional vaccines (Sekhon et al., 2014) in terms of inducing both cell-mediated and antibody-mediated adaptive immunity in humans, as well as memory response induction. (Bhardwaj et al., 2020). Nanovaccines provide effective immunization by improved targeting and antibody response activation at the cellular level (Sekhon et al., 2014). This is happening as nanotechnology has aided in the development of effective vaccine delivery mechanisms that protect the encapsulated antigen from the aggressive in vivo environment whilst also allowing for a sustained release (Sekhon et al., 2014) to antigen-presenting cells(APCs) (Kim et al., 2014) that aids in the induction of the vaccine's immunostimulatory properties (Sekhon et al., 2014). They are even more comfortable to use and nasal drops can be used as a delivery method of nanovaccines. Although other vaccines, such as those based on DNA, have performed well in animal models, they have performed

poorly in humans, especially in terms of preventing disease transmission. However, nanovaccines have improved our understanding of how the human body functions and what we can do to treat diseases. Nanovaccines would therefore be less expensive than traditional vaccination methods (Sekhon et al., 2014).

A number of vaccines nanocarriers have been designed and tested for their efficacy in transmitting antigens and adjuvants to immune cells in order to foster a healthy immune response (Kim et al., 2014). Aluminum was the first adjuvant used in vaccine formulation to boost antibody production, but it declines to provide strong cell-mediated immunity and has the risk of autoimmunity and long-term brain inflammation, which can lead to severe health problems and adjuvants have been reported to cause both local as well as systemic toxicity (Vijayan et al., 2019). On the other hand, due to the several beneficial advantages, such as improved antigen stability, targeted delivery, and long-term release, nano-scaled materials, like virus-like particles, liposomes, polymeric nanoparticles (NPs), and protein-based NPs, have received considerable attention as potential carriers for the delivery of vaccine antigens and adjuvants over the past decade. Antigens/adjuvants are either encapsulated within, or decorated on the NP surface (Vijayan et al., 2019).

Dendrimers, polymeric nanoparticles(NPs), metallic NPs, magnetic NPs, and quantum dots are examples of nanoparticles (NPs) that have proven to be effective vaccine adjuvants for infectious diseases and cancer therapy (Sekhon et al., 2014). Because most synthetic nanoparticles lack particular cell receptor binding sites, they interact structurally rather than functionally with Antigen Presenting Cells (APCs) to promote internalization. Protein-based nanoparticles, on the other hand, have the ability to interact structurally and functionally, since

they may store antigens and interact with pattern-associated receptors on APCs (Bhardwaj et al., 2020).

Nanovaccines can cause both cell-mediated and antibody-mediated immunity, as well as a memory response, and the prolonged release of antigens from nanoparticle depots can cause enhanced stimulation for a long time, reducing the need for frequent booster doses. As a result, nanovaccines can be used as both prophylactic and therapeutic, meaning they are given before or after the onset of the disease. Many nanovaccines are in various stages of clinical trials and have received FDA clearance (Bhardwaj et al., 2020).

There is inadequate research on the potential of prophylactic nanovaccines to prevent influenza viruses, as well as their advantages and downsides. As a result, in order to fill the gap, this research will examine the effect of prophylactic nanovaccines in activating several types of immunity, with the objective of minimizing the need for booster doses as vaccinations for the influenza disease.

1.8 Aim and Objectives

Aim of the study

The aim of this review is to compile the uses and benefits of nanovaccines over the traditional vaccines to prevent the influenza virus pandemic, as well as their obstacles.

Objective of the study

The primary objectives of this study are-

i. To accumulate as much knowledge from literature sources as possible about prophylactic applications of nanovaccines and its potential against influenza viruses.

ii. To determine the research opportunities for future researchers.

Chapter 2

Methodology

A research article discusses the existing state of information about a particular topic or idea. It typically summarizes previously written publications or papers on a specific topic of academic journals. Different types of literature reviews, such as structured and systematic reviews, may be used in a literature review framework. This research is a systematic literature review that provides an analysis of previous studies and interprets literature on a particular subject.

The research was conducted by scheming and screening a selection of prestigious publications and research papers. In terms of designing a review paper on applications and advantages of nanovaccines, various research databases, such as Science Direct, ResearchGate, PubMed, Google Scholar, Mendeley, Elsevier, Academic journals, Wiley Online Library etc. were used. Moreover, Mendeley by Elsevier is being used to cite the papers that were used.

Chapter 3

Results and Discussion

3.1 Mechanism of action of Human Influenza virus

Influenza viruses have claimed many lives since ancient times and remain a serious threat to mankind (Sangawa et al., 2013). Human infection by influenza viruses initiates in the respiratory tract and in most cases, infection is contained within this organ. Influenza virus that enters the host through the oral or nasal cavities is first countered by the mucus that covers the respiratory epithelium. If the virus is successful in getting through the mucous layer, it must next attach to and invade the respiratory epithelial cells. The virus can then spread to both non-immune and immune cells in the respiratory system like dendritic cells (DCs) and macrophages (Iwasaki & Pillai, 2014).

After entering into the body, influenza viruses with the help of Hemagglutinin (HA) (J. R. Chen, Liu, Tseng, & Ma, 2020) bind to the sialic acid of glycoproteins on the cell surface of the target cell (Sangawa et al., 2013). Human influenza HA binds to alpha-2,6 linked sialic acids, which are widely present on epithelial cells in the human upper respiratory tract (J. R. Chen et al., 2020). Influenza viruses are then endocytosed into the cell, where they release virus RNA and RNA-bound protein complexes Ribonucleoprotein (RNP) into the cytoplasm (uncoating). RNP released into the cytoplasm enter the nucleus, where they are transcribed and reproduced by the influenza virus's RNA-dependent RNA polymerase (RdRp). Subsequently, translated virus proteins, i.e., hemagglutinin, neuraminidase, and M2 proteins, migrate with the nascent RNP to the cell surface. Virus particles are then constructed and released from the cell membrane by neuraminidase (Sangawa et al., 2013) so they are then free to infect other cells,

which can be prevented by NA inhibitors (Figure 1) (Herold, Becker, Ridge, & Budinger, 2015). Moreover, Influenza B viruses are morphologically similar to Influenza A Virus (Herold et al., 2015).

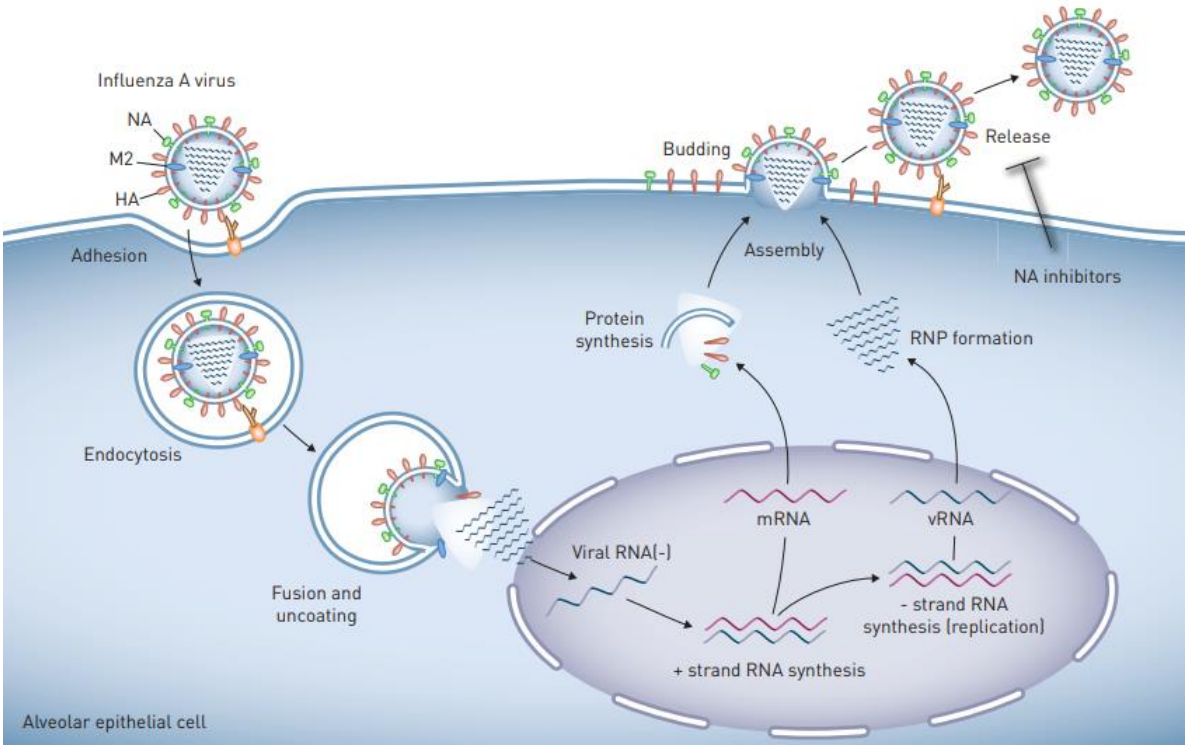

Figure 1. Mechanism of action of the Influenza A virus in the human lung epithelial cell (Herold et al., 2015).

3.2 Mechanism of Nanovaccines through Different routes and delivery

Vaccination is a powerful tool in the fight against influenza (Waithman & Mintern, 2012). Since the 1940s, vaccines that predominantly produce neutralizing antibodies against the viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) have substantially decreased the incidence of disease following infection and saved many lives (Sekiya et al., 2021). However, still there are chances of pandemic due the changes in the influenza virus variants. To give efforts are critically important to improve current regimes (Waithman & Mintern, 2012). In this situation, nanomedicine plays a key role in improving influenza vaccine

development (Asadi & Gholami, 2021). In order to do this, the mechanisms that underlie immunity to Influenza virus need to be studied in detail (Waithman & Mintern, 2012). Nanovaccines can be administered in a variety of ways to aid in the enhancement of immune responses. Prophylactic nanovaccines are usually given intranasally, intramuscularly, or subcutaneously (Bhardwaj et al., 2020).

3.2.1 Intranasal nanovaccines for Influenza

An intranasal vaccine, which neutralizes IgG, mucosal IgA, and T cell responses, triggers a strong immune response. Because of its similarity to the common route by which influenza viruses invade the host, the production of secreted IgA antibodies in large quantities, which could broaden immune responses and add to the cross-protective effects, and the induction of T-cell responses at mucosal sites, an intranasal influenza vaccine could be a powerful tool for protecting populations against the influenza virus (Qi et al., 2018). Additionally, intranasal vaccination produces humoral and cellular immune responses at the systemic level as well as on mucosal surfaces, making it more effective in protecting against respiratory viruses such as influenza.(Al-Halifa, Gauthier, Arpin, Bourgault, & Archambault, 2019).

The primary site for the induction of nasal immunity against administered vaccines is the nasal-associated lymphoid tissues (NALT). B cells, T cells, macrophages, and dendritic cells (DCs) are covered by epithelial cells, and a limited number of specialized microfold cells (M cells) make up NALT (Agrahari & Mitra, 2016). At first, M cells transfer particulate-based antigens from the apical side of the lumen to DCs or B cells, whereas soluble antigens are passively absorbed from the apical side of the epithelial membrane toward the basolateral region (Figure. 2) (Agrahari & Mitra, 2016). Once antigens are captured by DCs, they migrate to nearest lymphoid follicles or lymph node to present antigens to CD4+ T cells in the subepithelial region

(Neutra & Kozlowski, 2006). DCs-stimulated CD4+ T cells activate naïve B cells and undergo isotype switching to form antigen-specific IgA+ committed B cells. These committed IgA+ secreting B cells express high levels of CCR10 (mucosal homing receptors) and migrate from lymph nodes to the blood circulation. The circulating IgA+ B cells exit the blood and the expressed CCR10 in IgA+ B cells eventually aid to enter toward the effector sites in the nasal epithelial cells that express CCL28, a mucosa-associated epithelial chemokine (Figure. 2) (Agrahari & Mitra, 2016).

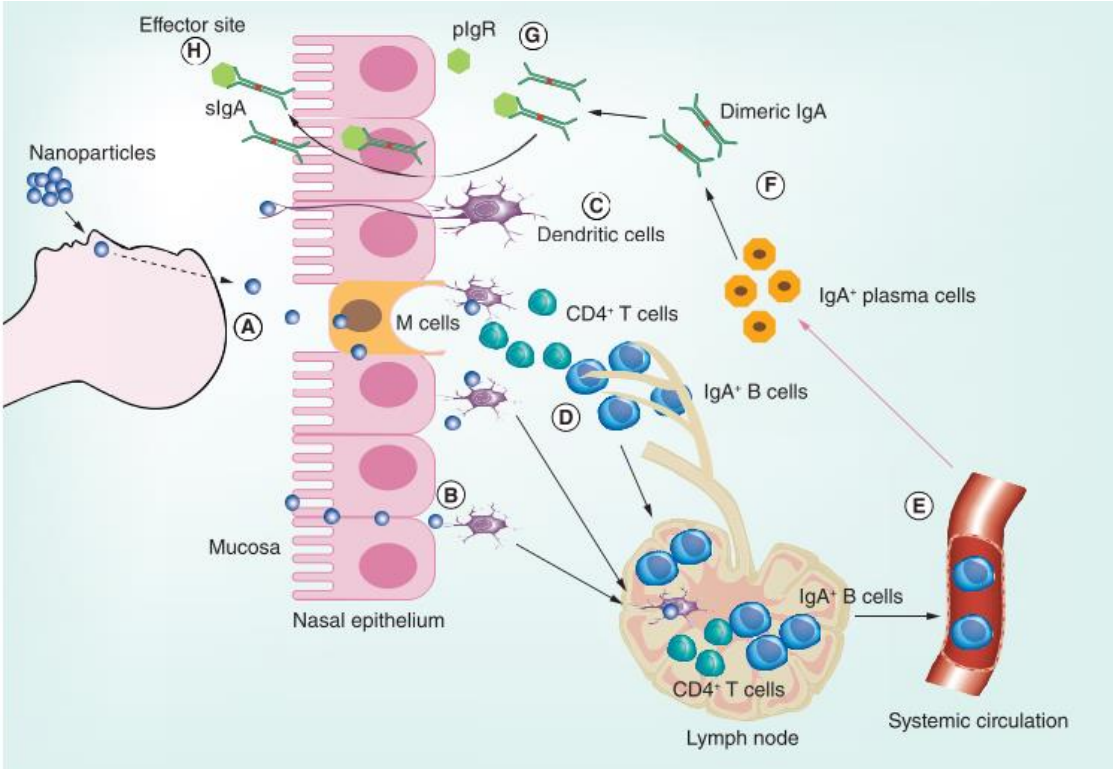

Figure 2. Mechanism of action of nasal nanovaccines (Agrahari & Mitra, 2016).

Abbreviations- DC: Dendritic cell; IL: Interleukin; M-cell: Microfold cell; pIgR: Polymeric immunoglobulin receptor; sIgA: Secretory IgA; Th: T-helper cell.

After that, IgA+ B cells undergo final differentiation and maturations to generate IgA+ plasma cells, which is a process enhanced by IL-5 and IL-6, a subset of T-helper 2 (Th2) cells, and

ultimately form dimeric or polymeric IgA. The dimeric or polymeric IgA binds with Polymeric immunoglobulin receptor (pIgR) in the basolateral region to form Secretory IgA (sIgA) which is further transcytosed toward the apical side of luminal surface in the nasal passage. Secretory IgA (sIgA) antibodies prevent the access of the toxins or pathogenic micro-organisms by recognizing, binding or neutralizing them and this is how sIgA produces the first line of defense against pathogens (Figure 2) (Neutra & Kozlowski, 2006).

Also, they can remove antigens by blocking access to the epithelial receptor trapping them in mucus and preventing their adherence to mucus by mucociliary-mediated exclusions. Additionally, other than mucosal sIgA, nasal immunization can also induce systemic antibodies (IgG) in the peripheral lymphoid tissues just like parenteral vaccines. This feature makes the intranasal route highly efficient for the delivery of vaccines (Agrahari & Mitra, 2016).

Following this mechanism, a number of attempts have been undertaken to develop a nanovaccine for influenza. A novel M2e-based influenza nanovaccine (3M2e-rHF) was developed by showing 3-sequential repetitions of M2e on self-assembled human heavy chain ferritin cages (rHF). Strong M2e-specific humoral, cellular, and mucosal immune responses were elicited by intranasal immunization with 3M2e-rHF. Moreover, the 3M2e-rHF nanovaccine protected mice from a fatal infection with homo-subtypic human H1N1 virus and hetero-subtypic avian H9N2 virus (Qi et al., 2018). In the absence of an adjuvant, intranasal vaccination with 3M2e-rHF nanoparticles generates powerful immunological responses in mice, including high titers of serum M2e-specific IgG antibodies, T-cell immune responses, and mucosal secretory-IgA antibodies. The 3M2e-rHF nanoparticles also provide full protection against a fatal infection with the H1N1 and H9N2 viruses, both homo-subtypic and hetero-subtypic. M2e-specific mucosal secretory-IgA and T-cell immune responses may play

significant roles in the prevention of infection, according to a study of the mechanism of protection underlying the intranasal vaccination with the 3M2e-rHF nanoparticle. According to the findings, the 3M2e-rHF nanoparticle is a potential needle-free, intranasally delivered, cross-protective influenza vaccination (Qi et al., 2018).

In addition, FluMist/Fluenz® is an intranasal quadrivalent live attenuated influenza vaccine (LAIV), with vaccine virus replication occurring in the epithelia of the upper respiratory tract, resulting in generation of both humoral and cell-mediated immune responses (Dibben et al., 2021). The intranasal quadrivalent live-attenuated vaccine induces nasal mucosal influenza-specific immunoglobulin A (IgA) to the hemagglutinin of each of the four components of the vaccine, which is advantageous because the nose is the portal of entry of influenza virus (Mossad, 2003).

Another study found that an intranasally administered polyanhydride-based IAV HA vaccine protects mice against H5N1 challenge infection by inducing virus neutralizing antibodies and cell-mediated immune responses (Renu et al., 2018).

In addition, when influenza vaccinations, which are generally 40nm particles, were given intranasally, both antibody and cytotoxic T-lymphocyte (CTL) responses were elicited in mice (Mossad, 2003).

3.2.2 Intramuscular nanovaccines for Influenza

The vaccination is administered into the muscle mass through intramuscular injection. To minimize adverse local effects, vaccines with adjuvants should be administered into muscle (Y.-C. Chen, Cheng, Yang, & Yeh, 2016). Also, in certain cases, the antigen may not be able to reach the Langerhans cells of the epidermis and thus intramuscular can be an alternate route

used as immune response produced can be modest due to its activity indirectly on dendritic cells (Yadav, Dibi, Mohammad, & Srouji, 2018). Additionally, the intramuscular route is favored due to the increased vascularity of muscle tissue and the corresponding increase in the bioavailability of drugs when administered intramuscularly (Soliman et al., 2018).

Several studies have shown that intramuscular route of nanovaccines can be efficacious. Like, a study reported that a single dose of recombinant intramuscular virus-like particles (VLPs) based vaccine against H5N1 influenza has resulted to be effective. The same study also stated that the use of microneedle vaccination of mice in the skin with a single dose of H1N1 influenza VLPs conferred protection superior to that with intramuscular injection (Bengalensis, Overview, Patil, & Patil, 2010). Another experiment outlined that a group of mice were given intramuscularly nanoparticles-based adjuvanted live-attenuated vaccine against H5N1 and it has shown effectful result (Raffael Nachbagauer et al., 2021).

Inflexal® V is a virosome-based influenza nanovaccine which is marketed in many countries and it should be given intramuscularly. A virosome is a vaccine delivery technique made up of a phospholipid membrane vesicle that contains viral-derived proteins and forms a pure fusion-active vesicle. Virosomes can only merge with target cells and cannot replicate within their hosts. In addition, virosomes are a novel, widely applicable adjuvant and carrier system that is utilized in numerous fields apart from conventional vaccination. (Y.-C. Chen et al., 2016).

3.2.3 Subcutaneous nanovaccines for Influenza

Through subcutaneous injection, nanovaccines migrate efficiently from the injection site into draining lymph nodes (Luo, Samandi, Wang, Chen, & Gao, 2017) and activate dendritic cells (DCs) as the antigen-bearing dendritic cells (DCs) can rapidly activate T cells in lymphoid organs to give the immune response (Zhang et al., 2020). To make this point clear, a study

demonstrated that as lymphatic capillaries in the skin have a diameter in the 10–80 mm range and would therefore be able to form a conduit for subcutaneously injected particles through virus-sized vaccine delivery systems like liposomes, virosomes, virus-like particles (VLPs), immune-stimulating complexes (ISCOMs) so that they can easily reach the draining lymph node. In addition, subcutaneous injected vaccines are able to make the interaction with DCs at the site of injection as well as within the lymph node (Scheerlinck & Greenwood, 2008).

Furthermore, another study found that subcutaneous vaccination of BALB/c mice with polyanhydride nanoparticles encapsulating H53 resulted in high titer neutralizing antibodies and increased CD4+ T cell numbers. As a result, vaccinated mice showed considerably greater levels of cell-mediated and antibody-mediated protective immunity than non-vaccinated animals. This nanovaccine was tested against H5N1 avian influenza, which has been identified to infect humans and is a major worldwide concern with the potential to become the next pandemic threat (K. A. Ross et al., 2015).

3.3 Designs of Nanovaccines for Influenza

In the production of vaccinations, adjuvants are occasionally added to vaccines in addition to vaccine antigens to increase immunogenicity. Adjuvants are required in the production of influenza vaccines for older people with weakened immune systems, as well as during pandemics when a rapid antibody response is necessary. Adjuvants are also needed in the development of new peptide-based influenza vaccines, which have a low immunogenicity (Khalaj-Hedayati, Chua, Smooker, & Lee, 2020).

The potential use of nanoparticles (NPs) as vaccine adjuvants has recently captivated interest. The use of nanoparticles in vaccine formulations has been shown to improve antigen stability, target antigen delivery, and assist delayed antigen release, reducing the need for booster doses.

Various nanoparticles (NPs) have been investigated in order to transfer antigens and boost immune responses against influenza antigens (Khalaj-Hedayati et al., 2020).

Nanomaterials can be used as adjuvants, immunogens, or nanocarriers, depending on their potential use (Bhardwaj et al., 2020). A nano vaccine is based on nano-sized particles whose composition can be proteic, lipidic, metallic, polymeric, or based on graphene served as antigen delivery vehicles (Karthikeyan, Laxmi Deepak Bhatlu, Sukanya, & Jayan, 2020).

Various types of nano-scaled material have been employed for the design and fabrication of nanovaccines based on nature or man-made nanocarriers. Bacterial spores, Proteosomes, exosomes, liposomes, virosomes, SuperFluids, nanoparticle-based nanobeads, virus-like particles(VLP) and bacteriophages are some instances of nano-sized vehicles that are used as potential nanocarriers for vaccine delivery to immune systems (Gill, 2013).

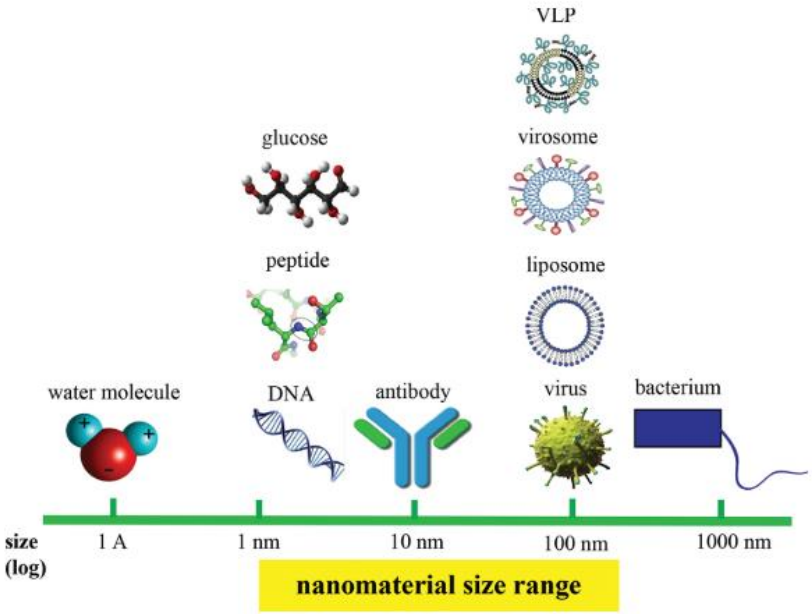

Figure 3. *Size comparison of nanomaterials used in nanotechnology (Y.-C. Chen et al., 2016).*

The physicochemical properties of NPs (e.g., size, shape, surface charge and chemistry, and ligand density) can be tuned and engineered to ease the biodistribution and therapeutic loading, site-specific targeting, and immunogenicity. The parameters that should be taken into consideration when designing nanocarriers for immunotherapy are summarized in the following figure (Fontana et al., 2019).

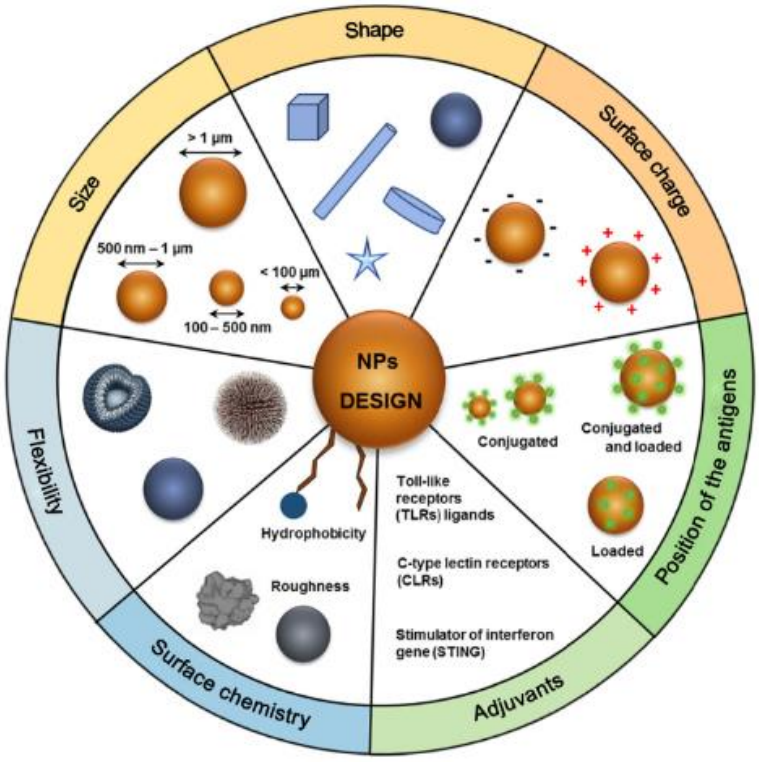

Figure 4. Different parameters for a rational design of nano-systems to enhance the delivery of immunotherapies, increase tissue permeation, and avoid nonspecific uptake (Fontana et al., 2019).

Nanoparticles can be used as adjuvants with specific antigens or as immunogen themselves. Vaccine antigens are either encapsulated inside or surface decorated over nanoparticles to get delivered efficiently (Figure 5) (Bhardwaj et al., 2020). Surface modification or encapsulation typically functionalizes with the antigen (Khalaj-Hedayati et al., 2020). Free antigens may degrade and provoke local immune responses at the administration site, thus,

encapsulating free antigens inside nanoparticles inhibits their degradation and allows for extended and regulated release at the target site. Apart from encapsulation, surface absorption, conjugation, and mixing have all been used to deliver antigens and nanoparticles together (Bhardwaj et al., 2020).

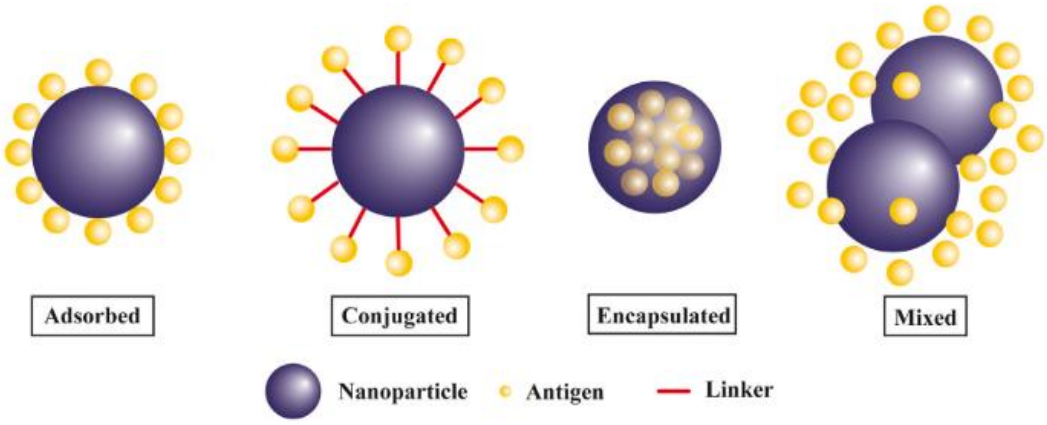

Figure 5. Design of nanovaccine (Bhardwaj et al., 2020).

3.3.1 Different approaches to design a nanovaccine for Influenza

A nanovaccine is designed using a variety of nanoparticles, each of which performs a particular function (Bhardwaj et al., 2020). Some approaches to design the influenza nanovaccines are discussed below.

3.3.1.1 Design of the self-assembling peptide and protein-based nanovaccine

Short peptide fragments or proteins with second or third structures make up peptide vaccines (Asadi & Gholami, 2021). In the formation of peptide nanoparticles, self-assembly is essential. These self-assembly protein nanoparticles have a size range of 20–100 nm (Petersen, Phanse, Ramer-Tait, Wannemuehler, & Narasimhan, 2012).

Ferritin is the outstanding example of self-assembled nanoparticles with intracellular iron storage as their principal function. The vaccine, which is based on ferritin, is thermostable and chemically stable. Influenza matrix protein 2(M2e) cage intra-nasal nanovaccines based on self-assembling recombinant human heavy chain ferritin (rHF) cages are now in the preclinical stage against homo- and hetero-subtypic influenza viruses (Asadi & Gholami, 2021).

The ectodomain of matrix protein 2 (M2e) is the most conservative and protective viral antigen, capable of generating hetero-subtype immunity against various virus strains and subtypes (Ding et al., 2020). As a result, M2e has been a major focus in the development of universal influenza vaccines (Karthikeyan et al., 2020). Due to low immunogenicity, multicopy M2e are usually displayed on the surface of nanoparticles to constitute universal nanovaccines (Karthikeyan et al., 2020).

Nevertheless, an intranasal nanovaccine targeting the conserved ectodomain of influenza matrix protein 2 (M2e) has been established. M2e is provided in three-sequence repetitions (3M2e) on a self-assembling recombinant human heavy chain ferritin (rHF) cage to produce a new M2e-based influenza nanovaccine (3M2e-rHF) (Figure 6a) (Qi et al., 2018).

After rHF self-assembly, three consecutive repetitions of M2e peptides were attached to the N-terminus of human ferritin heavy chain (rHF) (Figure 6a, 6b), resulting in 3M2e-rHF fusion protein. Figure 6c shows a schematic of the 3M2e-rHF nanoparticle.(Qi et al., 2018).

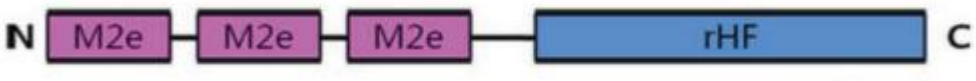

Figure 6a. Design of the 3M2e-rHF fusion protein (Qi et al., 2018).

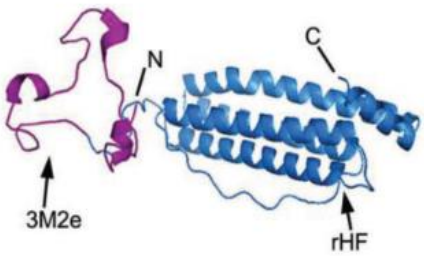

Figure 6b. The structure of a 3M2e-rHF monomer in three dimensions (3D) (Qi et al., 2018).

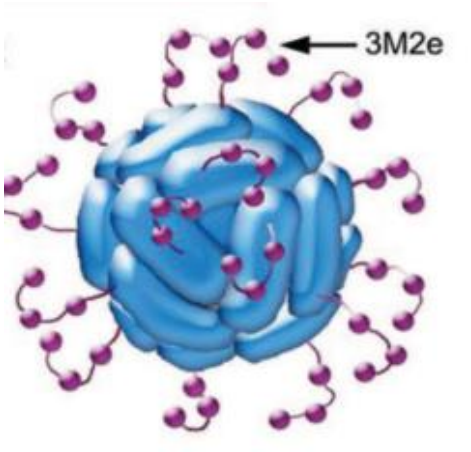

Figure 6c. Diagram of a 3M2e-rHF nanoparticle (Qi et al., 2018).

On top of the ferritin nanoparticle cage, there is another captivating protein nanoparticle called apoferritin (Aft) which is basically ferritin nanocage without iron. Apoferritin (Aft) composed of 24 subunits that assemble into a large spherical hollow cage with an outer diameter of 12 nm and inner diameter of 8 nm. It was used as a carrier to construct a biomimetic influenza vaccine. However, it was designed in a way where the nanocage was encapsulated with a conserved internal nucleoprotein (NP) antigen peptide and outer surface of the apoferritin was chemically conjugated with the surface antigen hemagglutinin (HA) protein (Wei, Li, Yang, Su, & Zhang, 2020). The process includes release of the NP peptide from the apoferritin (Aft) particles which were endocytosed by antigen-presenting cells (APCs), followed by presenting on a major histocompatibility complex (MHC) class molecules to elicit the virus-specific CD8+

CTL response, which aids in clearance of influenza infection and contributes to cross-protective efficacy against influenza. Moreover, the sustained antigen release from nanoparticle vaccines will also benefit stimulation of long-term effector memory cellular response (Wei et al., 2020).

3.3.1.2 Design of virosome-based nanovaccines

Virosomes are lipid vesicles with a diameter of around 150 nm (Y.-C. Chen et al., 2016) that contain virus-derived protein but lack the viral genome and internal proteins (Khalaj-Hedayati et al., 2020). Virosomes are nontoxic, biodegradable, and do not trigger antiphospholipid antibody reactions (Khalaj-Hedayati et al., 2020).

The membrane proteins can either be produced via recombinant technology or purified from the corresponding viruses. During surface protein purification, virus membrane is normally solubilized and reconstructed using mild detergents without causing denaturation (Khalaj-Hedayati et al., 2020).

In the preparation method of virosomes, an encapsulated virus is first solubilized by detergent, and then the nucleocapsid structure is extracted following ultracentrifugation. The sedimented viral proteins from the supernatants were isolated after the detergent was removed. Finally, hollow membrane vesicles are reconstituted using viral antigens epitopes and viral fusion peptide, which are relatively hydrophobic proteins, and lipid. Furthermore, the inclusion of virus components (RNA, DNA, or proteins) and antiviral drugs might enhance virosome pathogenicity (Figure 7) (Asadi & Gholami, 2021).

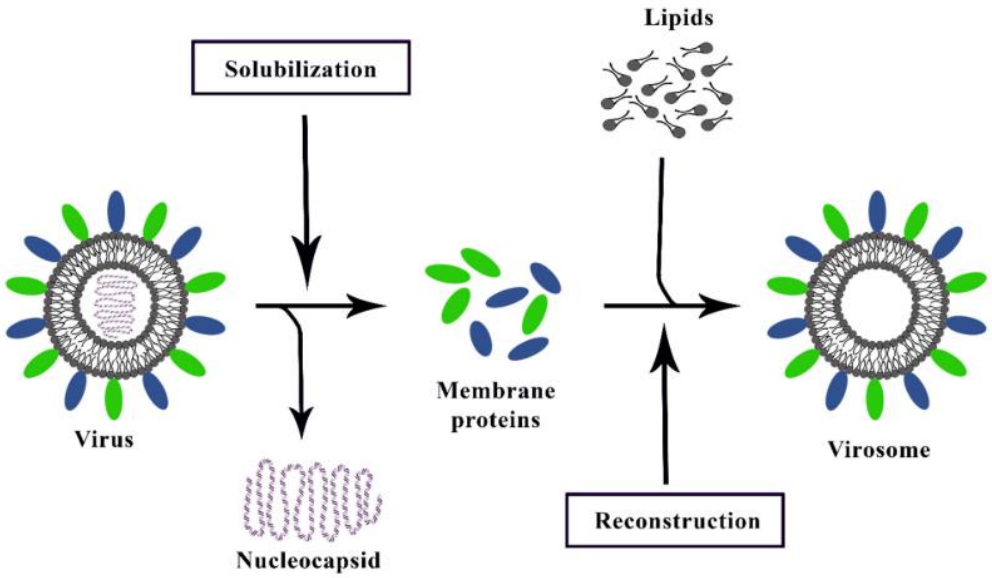

Figure 7. The current process of virosome preparation (Asadi & Gholami, 2021).

The influenza virus is the most widely utilized virus for virosome synthesis, with each virosome being around 150 nm in diameter on average (Khalaj-Hedayati et al., 2020). Influenza vaccinations based on virosome nanocarrier are a new FDA-approved technique that reduces viral morbidity and mortality (Asadi & Gholami, 2021).

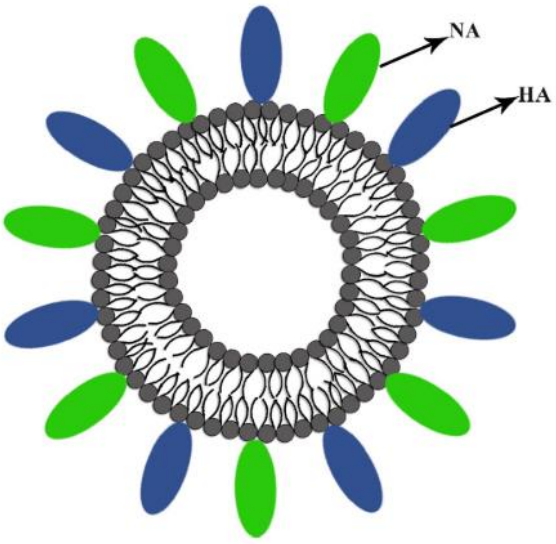

Figure 8. Schematic design of influenza virosome (Asadi & Gholami, 2021).

3.3.1.3 Design of Virus-Like Particles (VLPs)-based nanovaccines

Virus-like particles (VLPs) are nonreplicating, self-assembling particles (Khalaj-Hedayati et al., 2020) with spherical supramolecular assemblies of 20–200 nm diameter (Al-Halifa et al., 2019) that are devoid of infectious genetic material (Khalaj-Hedayati et al., 2020).

Virus-like particles (VLPs) serve as molecular carriers which are built in a symmetric manner from hundreds of viral capsid proteins. However, it provides a regular arrangement of the target peptides on the surface of the particle. Some factors of VLPs, like mimicking the formation of viral capsids, having a specific conformation and providing a high number of introduced peptides per particle, help inducing an immune response, binding of receptors, etc. (Blokhina et al., 2013).

VLPs may be made from a variety of cell lines, including bacteria, yeast, insects, and animals. Because of their immunogenic properties, including as their comparable size to the original pathogen, repeating surface geometry, and capacity to trigger innate and adaptive immune responses, they can be utilized as both particle carriers and immunopotentiators in vaccine production. The major benefit of VLP-based vaccinations is that the host's immune system can detect VLPs in the same manner it recognizes the original virus, resulting in a strong immunological response. They were developed largely to enhance B cell activation and generate strong antibody responses when T helper cells were activated. VLP-based methods are also being studied as a potential method for developing a universal influenza vaccine. To create an effective VLP-based vaccination, the most appropriate VLP structure must be chosen, and antigens must be added without compromising the VLPs' stability (Khalaj-Hedayati et al., 2020).

An investigation is done with VLPs containing influenza virus (H1N1) hemagglutinin (HA) and matrix (M1) proteins to examine their immunogenicity, long-term cross-protective efficacy, and effects on lung proinflammatory cytokines in mice. The investigation shows that Influenza VLPs induced mucosal immunoglobulin G and cellular immune responses against a lethal virus challenge with homologous as well as heterologous virus strains, which were reactivated rapidly upon further virus challenge. Also, long-lived antibody-secreting cells were detected in the bone marrow of immunized mice(Quan, Huang, Compans, & Kang, 2007).

Also, self-assembled VLPs nanovaacine, which consists of numerous copies of M2e protein inserted into capsid protein, provides immunity against IAV in both mice and pigs, according to another research (Jin & Deng, 2019). Immunization with an VLP named Tandiflu1 that is containing 4 conserved antigens from matrix protein 2 ectodomain and hemagglutinin stalk, leads to production of cross-reactive and protective antibodies. The polyclonal antibodies induced by Tandiflu1 can bind IAV Group 1 hemagglutinin types H1, H5, H11, H9, H16 and a conserved epitope on matrix protein 2 expressed by most strains of IAV (Ramirez et al., 2018).

3.4 Formulation of Nanovaccines

Nanovaccines are formulated either by physical adsorption, chemical conjugation, encapsulation or physical mixing of antigen with nanoparticles (Bhardwaj et al., 2020). Moreover, lipids, proteins, metals, polymers, and other organic components are used as nanocarriers in vaccine formulations using different high-throughput methods (Asadi & Gholami, 2021)

There are four popular nanotechnology-based vaccines for different diseases that have been on the market for over ten years in various countries. Inflexal® V is one of them, and it's used to treat influenza. As a delivery and adjuvant system, they employ nanoparticles-virosomes (special liposomes) or virus-like particles (VLPs), which should all be given intramuscularly (IM) and kept at 2–8°C (Y.-C. Chen et al., 2016). Inflexal® V is a trivalent influenza virus virosome vaccine made up of three monovalent virosome pools (Herzog et al., 2009), each of which includes an inactivated version of two A virus strains and one B virus strain, as well as the influenza virus's particular antigen HA and NA subunits (Asadi & Gholami, 2021).

Product	**Inflexal® V**
Indication	Influenza
Formulation	A combination of three monovalent virosome pools
Nanoparticle	Virosome
Size in diameter	150 nm (100–300 nm)

Table 1. Formulation of the Inflexal® V nanovaccine (Y.-C. Chen et al., 2016).

Additionally, Flumist® is an intranasally administered vaccine, a live attenuated influenza vaccine (LAIV), underlines the potential of mimicking a natural influenza infection able to trigger protective mucosal immune response (Quan Le et al., 2020). FluMist is a intranasal spray (Y.-C. Chen et al., 2016) live attenuated influenza vaccine (LAIV) that uses novel technology allowing administration of the vaccine via a noninvasive, intranasal route (Carter & Curran, 2011).

These vaccines are usually available as trivalent or quadrivalent formations, containing recent influenza A virus strains of the H1N1 and H3N2 subtypes in combination with one or two influenza B virus strains (Sekiya et al., 2021).

Three live influenza viruses are included in the trivalent Flumist: two A strains (subtypes H1N1 and H3N2) and one B strain (Carter & Curran, 2011). In addition, quadrivalent Flumist has four viral strains in its composition (a type A/H1N1, a type A/H3N2, and two type B strains) (Y.-C. Chen et al., 2016).

3.5 Prophylactic nanovaccines for influenza

While the immunogenicity and efficacy of influenza HA in the prevention of seasonal influenza is well established, gaps exist in the protection of the elderly and questions remain regarding the optimal approach and formulations for protection against pandemic strains (Galloway et al., 2013). Prophylactic vaccines are developed to fill those gaps. At present, antibody-mediated protective responses against hemagglutinin and neuraminidase are produced by two kinds of influenza vaccinations based on strain A and strain B. Due to error-prone RNA dependent RNA replication in influenzas, these antigens are especially susceptible to antigenic shift and drift (Bhardwaj et al., 2020).

As the nanotechnology field grows, its applications in anti-influenza therapies and prevention methods are expanding. Intensive research on nanotechnology-based antivirals and their influence on the viral life cycle and cell itself broadens the knowledge concerning virus biology, and contributes to the development of nanomedicines. Nanotechnology trends towards the facilitation of new methodologies applying NPs against influenza viruses (Applications et al., 2020).

3.5.1 Prophylactic action of nanovaccine

Different types of nanovaccines can enhance antigen immunogenicity by enhancing immunogenic responses and establishing memory against antigens at low dosages.

Prophylactic vaccinations, for example, are provided before the onset of disease with the goal of developing protective immunity (Bhardwaj et al., 2020). To develop immunogenic memory against particular diseases is the objective of prophylactic vaccines (Bhardwaj et al., 2020) as successful prophylactic vaccines must induce antigen-specific memory B cells capable of rapidly proliferating upon antigen stimulation (Huntimer et al., 2013).

Prophylactic nanoparticle vaccine is designed to produce strong and long-lasting immune response in the human body and ideally, an prophylactic influenza vaccine is supposed to give protection against all strains (Soema, Kompier, Amorij, & Kersten, 2015). It may produce both cellular- and humoral-mediated immunity. To produce immunity, the nanovaccine with specific strains endocytosed into antigen-presenting cells (APCs) and activate dendritic cells (DCs) to migrate them to lymph nodes. The activated DCs then deliver antigens to CD4+ T-helper cells (Th cells) via the MHC-II receptor on CD4+ T-helper cells (Th cells). Following that, Th cell activation and maturation result in the production of a number of cytokines signals, which cause Th cells to split into Th1 and Th2 cell subsets. Here between two types of Th cells, the Th1 subset secretes the most proinflammatory cytokines, which promote the proliferation of cytotoxic T lymphocytes (CTLs) and improve cell-mediated immunity. Th2 subsets, on the other hand, produce cytokines that drive B cell proliferation during antibody-mediated immune responses (e.g., IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13). This is how any prophylactic nanovaccine's cell-mediated and antibody-mediated immune responses are balanced by Th1 and Th2 subsets (Bhardwaj et al., 2020).

3.5.2 Examples of some prophylactic influenza nanovaccines

Nano vaccines have been widely experimented as prophylaxis of important diseases like influenza (Zaheer, Pal, & Zaheer, 2021). The novel prophylactic influenza nanovaccines under

development aim at overcoming the shortcomings associated with current vaccines, including the limited efficacy resulting from their strain specificity, the limited production capacity and so on (Ben-Yedidia & Arnon, 2007).

Several influenza nanovaccines are under different clinical phases and some got FDA approval to be marketed. Among them, Inflexal® V is a trivalent subunit nanovaccine which follow virosome-based nanocarrier delivery (Mischler & Metcalfe, 2002) and is proved to be highly efficacious in people for all age groups and shows a good immunogenicity in both healthy and immunocompromised elderly, adults and children (Herzog et al., 2009). In contrast, FluMist is a quadrivalent live attenuated influenza vaccine (QLAIV) which is administered intranasally and four strains of influenza type A and B strains (Dibben et al., 2021).

Name	Type of nanoparticles	Use	Company	Approval stage
Inflexal V	Virosomes	Subunit Influenza vaccine	Crucell, Berna Biotech	FDA approved
FluMist	N-trimethyl chitosan-mono-N-carboxymethyl chitosan nanocomplexes	Quadrivalent live attenuated influenza vaccines	Medimmune	FDA approved

Table 2. Some clinically used influenza nanovaccines (Yadav et al., 2018).

3.6 Comparison between current uses and potential use in Influenza vaccines

Antigenic drift and shift results in the development of new strains, which is followed by cross-immunity-mediated competition between antigenically similar strains, leading in the gradual replacement of old strains with new variations (J. R. Chen et al., 2020). Current influenza vaccines, such as inactivated and live-attenuated vaccines, are strain-specific and induce protection by activating an immune response against the strain-specific influenza

hemagglutinin protein (HA), and appear to have such a narrow range of coverage, showing extensive surveillance against predicted strains, and are required to vaccinate annually as circulating strains constantly changing throughout time (Wang et al., 2020) (J. R. Chen et al., 2020). Until now, cross-protective influenza vaccines have been developed using a variety of conventional vaccinations, including inactivated vaccines, subunit vaccines, and DNA vaccines. However, the current cross-protective influenza vaccines, have a number of drawbacks, including low immunogenicity, low hetero-subtypic protective effectiveness, and limited appropriateness for large-scale production (Qi et al., 2018).

For instance, whole pathogen-based vaccines were the most popular, mostly because of the simplicity and relatively low cost of their production. However, the use of whole pathogen-based vaccines comes with some demerits such as chances of autoimmune or strong allergic responses, production difficulties (not all pathogens can be cultured), biological contamination, etc. Therefore, alternative methods using subunit antigens such as toxins or small-pathogen fragments (proteins, carbohydrates and peptides) provide a rational approach toward safe immunizations. Unfortunately, induction of the desired immune responses with subunit-based antigens is often difficult to achieve even after parenteral immunization, as they are poorly immunogenic due to the lack of danger signals (Agrahari & Mitra, 2016). On that account, subunit-based vaccines require adjuvants to induce humoral and cellular immune responses (Huntimer et al., 2013).

Some current seasonal influenza virus vaccines are developed that contain three or four strains of influenza virus that cover the viruses circulating in the human population. The vaccine strain composition of these vaccines is based on a prediction and mismatches occur relatively frequently due to the increased antigenic diversity. Therefore, the development of an universal

influenza virus vaccine that could protect against all influenza viruses is a major focus area for the research community (Raffael Nachbagauer et al., 2021).

In that case, by displaying cross-protective antibodies on nanoparticles and increasing their immunogenicity, nanotechnology may provide prospects for the creation of cross-protective influenza vaccines. Antigen presentation and immune activation have been demonstrated by many natural protein nanoparticles. (Qi et al., 2018). Several next-generation influenza vaccines are being developed with the objective of broadening or extending the human immune response with novel antigens and adjuvants, gradually expanding the strain-specific nature of current vaccines to include all strains within a subtype (e.g. all H1 strains), multiple subtypes (e.g. H1/H5/H9), or incorporating all subtypes within a group (influenza A group 1 or group 2), with the ultimate goal of developing a genuinely "universal" pandemic influenza vaccine that can elicit lifelong protection against all influenza A and B viruses (J. R. Chen et al., 2020). In the manufacturing of vaccines, nanomedicine plays a crucial role. Cost-effectiveness, improved vaccine stability, encapsulated antigen protection from premature degradation, no need for booster doses, excellent adjuvant properties, temporal and spatial target delivery, and a strong innate immune response in humoral and cellular levels, and discovery of desired vaccines for hard-to-treat viral disease are some of the critical characteristics of nanocarriers in biomedical sectors, particularly vaccine design (Asadi & Gholami, 2021).

The live, attenuated influenza vaccine (LAIV) is sprayed into the nostrils and contains no thimerosal or preservatives. Since it is given through the natural point of entry of the influenza virus, LAIV induces a considerably greater immune response than inactivated vaccines. Furthermore, this intranasal spray vaccination can prevent 50% more cases than IM-administered flu injections in younger children (Y.-C. Chen et al., 2016).

3.7 Current advancements in development of Influenza Viruses

The effectiveness of the vaccine is largely dependent on how well matched the vaccine strains are with the circulating influenza virus strains (Sekiya et al., 2021). Even through, influenza vaccines are considered to form the main prophylactic measure against pandemic influenza (Leroux-Roels & Leroux-Roels, 2009), due to the antigenic variability of the influenza viruses, previously administered vaccines produced antibodies against some specific known strains can no longer able to be neutralized effectively (Y.-C. Chen et al., 2016).

In different countries, three kinds of influenza vaccinations are now approved for human use: “inactivated,” “cold-adapted “live attenuated,” and “recombinant HA”. These vaccines are usually available as trivalent or quadrivalent formations, containing recent influenza A virus strains of the H1N1 and H3N2 subtypes in combination with one or two influenza B virus strains of the Yamagata and Victoria lineages (Sekiya et al., 2021).

Additionally, several pandemic vaccines have been developed in the preparation of possible future outbreaks of highly pathogenic influenza strains (Soema et al., 2015).

During the last decade, Inflexal® V has shown an excellent tolerability profile due to its biocompatibility and purity. The vaccine is made up of three monovalent virosome pools, each composed of the HA and NA glycoproteins of a single influenza virus strain (Y.-C. Chen et al., 2016) and contain no thiomersal or formaldehyde and its purity is reflected in the low ovalbumin content. By mimicking natural infection, the vaccine is highly efficacious and shows a good immunogenicity in both healthy and immunocompromised elderly, adults and children (Herzog et al., 2009). Wherefore, the product has been approved by many countries and is the only adjuvanted influenza vaccine licensed for all age groups (Y.-C. Chen et al., 2016).

Moreover, few nasal influenza vaccines for human use have been licensed and commercialized. The best known are FluMist (Medimmune) quadrivalent live attenuated influenza vaccines (Bernocchi, Carpentier, & Betbeder, 2017). For the prevention of influenza, quadrivalent intranasal spray attenuated vaccination is available with four viral strains (type A/H1N1, type A/H3N2, and two type B strains) (Ali et al., 2004).

With the development of noble viruses, pandemic diseases can emerge, posing a threat to both humans and animals. As a result, many vaccinations are being developed to protect people from these viruses in order to lower death rates (Yadav et al., 2018).

As discussed, several approaches are made to make a better influenza vaccine. Among all, some studies described nanoparticles showed a tremendous response and many of these are under development. Some of these approaches are outlined in the following table-

Nanocarrier	**Vaccine candidate composition**	**Clinical Phase**	**Reference**
Liposome	H1N1 split virus	Phase I	(Bhardwaj et al., 2020)
Virus-Like Particles (VLP)	A mix of Influenza A (H7N9) VLP Antigen (HA, NA, M1)	Phase I	(Khalaj-Hedayati et al., 2020)
Virus-Like Particles (VLP)	Influenza VLP-HA (H1, H8, H13, H3, H4, H10)	Preclinical	(Khalaj-Hedayati et al., 2020)
Human ferritin Cage	M2e protein	Preclinical	(Bhardwaj et al., 2020)

Gold nanoparticles	Extracellular portion of M2 protein (Influenza virus)	Preclinical	(Bhardwaj et al., 2020)
Proteosomes	Different protein complex	Preclinical	(Yadav et al., 2018)
Virus-Like Particles (VLP)	Recombinant hemagglutinin (HA) protein	Phase III	(Nooraei, Bahrulolum, Hoseini, Katalani, & Hajizade, 2021)
Virus-Like Particles (VLP)	A mix of recombinant H1, H3, and two B hemagglutinin proteins	Phase III	(Nooraei et al., 2021)

Table 3. Some examples of nanoparticle-based influenza vaccines currently being developed.

3.8 Benefits of nanovaccines for Influenza over current technology

As we have already discussed that the platform of nanotechnology in case of vaccination offers several distinct advantages: the particles degrade into biocompatible products, activate APC, maintain the stability of encapsulated antigen, enable dose sparing of the antigen, and may be stored at room temperature or higher for up to four months thus breaking the cold chain. Another important feature of our nanoparticle technology is that it provides a sustained release of encapsulated antigen via surface erosion and acts as a long-term antigen depot. Therefore, it could mimic the antigen depot that occurs after IAV infections and potentiate tissue-resident memory formation (Zacharias et al., 2018).

The conventional kinds, on the other hand, comprise either inactivated or live-attenuated viruses or viral protein subunits. Therefore, there might be seen various shortcomings of

conventional vaccinations like weak cell-mediated immune responses, safety warnings of viral infections in vaccinated persons, the need for booster doses, and the onset of immunogenicity.

On the other hand, the conventional types contain either inactivated or live-attenuated viruses or protein subunits of viral particles. Conventional vaccines have some deficiencies include weak cell-mediated immune responses, safety alarm of viral infections in vaccinated individuals, requiring booster doses, and the emergence of immunogenicity. Furthermore, sufficient data demonstrates that conventional influenza vaccinations will not provide significant protection against a future pandemic (Asadi & Gholami, 2021). Even though, due to environmental selection and antigenic variations, chances of influenza epidemic still persist. Although animal influenza virus is distinct from the human virus, zoonotic animal viruses can still occasionally infect humans through direct or indirect contact. Avian and swine influenza viruses have been known to infect humans in some countries. Thus, there is an unmet need for producing more efficient vaccines that can provide both antibody-mediated and cell-mediated immunity to confront homologous and heterologous variants (Bhardwaj et al., 2020).

To overcome all the drawbacks of the conventional vaccines, nanoparticles employed in the vaccine development. In the figure, it is shown that nanoparticles may aid in the storage duration extension, stability at room temperature, investigation of alternate administration routes, and assistance of sustained delivery when vaccines encapsulated in nanoparticle which are polymers in solid form (Y.-C. Chen et al., 2016).

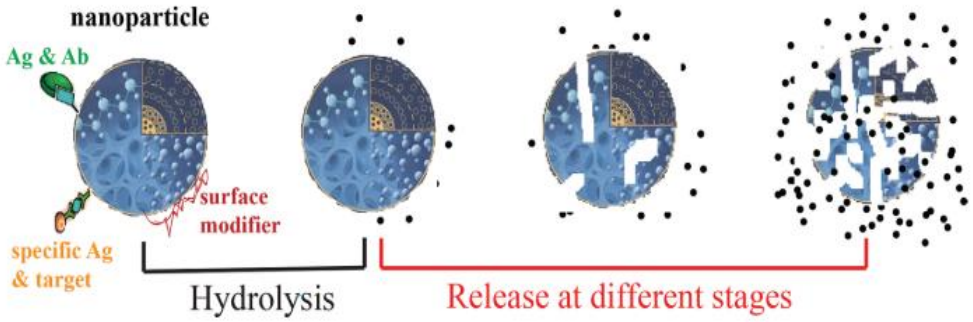

Figure 9. Vaccines in nanoparticle (Y.-C. Chen et al., 2016).

Moreover, conventional vaccines generally required many boosters to get desired effect, whereas, nanovaccines may only require to be boosted once (Y.-C. Chen et al., 2016).

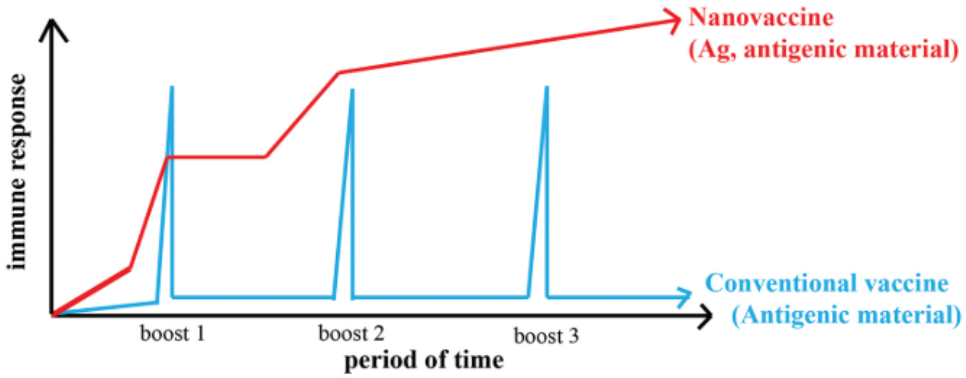

Figure 10. Comparison of vaccine boost between conventional vaccine and nanovaccine (Y.-C. Chen et al., 2016).

Furthermore, soluble antigens produced by nanovaccines have the potential to promote both humoral (B cell responses) and cell-mediated immunity (helper and cytotoxic T cell responses) (Figure 11) (Y.-C. Chen et al., 2016).

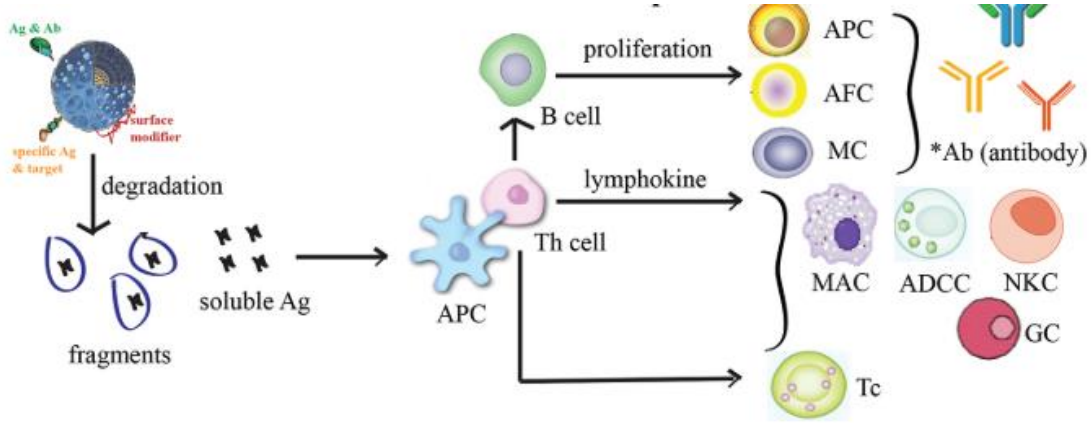

Figure 11. Interaction of nanovaccines with the immune responses (Y.-C. Chen et al., 2016).

*Ab (antibody): serum immunoglobulin G (IgG), mucosal IgG and mucosal IgA.

Abbreviations: antigen (Ag); antibody (Ab); antigen presenting cell (APC); antibody forming cell (AFC); antibody-dependent cytotoxic cell (ADCC); cytotoxic T cell (Tc); granulocyte (GC); helper T cell (Th); macrophage (MAC); memory cell (MC); natural killer cell (NKC) (Y.-C. Chen et al., 2016).

In addition, the potential of a combination nanovaccine that included both recombinant hemagglutinin and nucleoprotein to protect against seasonal influenza virus infection was investigated. In comparison to each adjuvant alone, vaccine formulations containing two nano adjuvants, polyanhydride nanoparticles and pentablock copolymer micelles, were demonstrated to improve challenge protection (K. Ross, Senapati, Alley, & Darling, 2019).

Furthermore, low vaccine efficacy in naïve populations such as young children, or in the elderly, who possess weakened immune systems, indicates that influenza vaccines need to be more personalized to provide broader community protection (Sekiya et al., 2021). For instance, Inflexal® V, a novel virosome-based trivalent influenza nanovaccine, has been shown to be highly immunogenic and well tolerated in children, young adults, and the elderly (Mischler & Metcalfe, 2002).

Also, nanovaccines are safe and well tolerated than conventional vaccines. For instances, Inflexal® V contained less than 10ng of ovalbumin per dose, satisfying the European Pharmacopoeia requirements for virosomal influenza vaccines by far. In contrast, conventional influenza vaccines are permitted to contain up to 1 microgram ovalbumin per dose. As a result, an influenza vaccine with this low ovalbumin content is expected to induce less allergic reactions (Herzog et al., 2009).

As a result, using the platform of nanotechnology to produce vaccines with high immunogenicity, cross-protection, and ease of administration, as well as being cost-effective and suited for large-scale manufacturing, might be an excellent strategy (Qi et al., 2018).

3.9 Challenges to formulate the nanovaccine

Some challenges must be addressed in the use of nanoparticles as vaccines, including stability during manufacture and storage, nonthermal sterilization, and formulation repeatability in large-scale production. One of the key challenges is reproducibility of formulation throughout manufacturing. When evaluating size-dependent immunogenicity, for example, a research found that just altering the size and shape of nanomaterials can vary their toxicity, even if the composition stays unchanged. Additionally, the nanoparticles may cause toxicity if they are removed slowly over a lengthy period of time. Furthermore, even while nanoparticles may be eliminated rapidly from the body, bigger equivalents may aggregate in some major organs which can cause toxicity (Y.-C. Chen et al., 2016).

The researchers' current challenge is to design nanoparticle-based formulations capable of encapsulating and releasing biologically functional protective antigens against all the strains of influenza virus that would result in a robust, high avidity, neutralizing antibody response after a single administration (Petersen, Phanse, Ramer-Tait, Wannemuehler, & Narasimhan, 2012).

Moreover, due to low yields in two strains, AstraZeneca's LAIV product FluMist faced production problems in 2019, resulting in a decrease in shipments globally (J. R. Chen et al., 2020).

Besides, the detergents applied in the preparation of virosome have a significant impact on the effectiveness of fusogenic virosomes. However, low-CMC detergents such as octaethylene glycol mono (n-dodecyl) ether (C12E8), Triton X-100, nonidet p-40, and others are difficult to remove after solubilization. High-CMC detergents, such as the nonionic octylglucoside (OG), result in inactive virosomes, which are then replaced by another detergent (Asadi & Gholami, 2021).

Chapter 4

Future Studies

Despite the fact that several investigations and experiments have been conducted in order to produce a universal influenza vaccine, there are still numerous hurdles and obstacles to overcome. To address these issues, more research might be conducted in the future.

Future researchers may investigate developing a universal influenza vaccine based on the nanotechnology platform that is effective against all the influenza strains, has a long-lasting and powerful impact after a single dose. Additionally, greater emphasis should be placed on the antigenic variability of influenza virus concept and work on that point.

Furthermore, it is essential to consider the possible impacts of nanovaccines on human body through assessing the impact of cell growth and death, gene expression in the body when different cell types are introduced to nanoparticles. Since, nanomaterials can be hazardous sometimes, therefore the safety and quality control of nanovaccines should be studied with greater caution in the future to protect the safety of human health and efforts should be made to induce nontoxic nanoparticles.

Chapter 5

Conclusion

Nanotechnology has resulted in an enormous revolution in vaccination, attempting to address all of the shortcomings that conventional vaccinations have. Nanovaccines has showed its efficacy against influenza virus that has various subtypes. However, nanotechnology has been trying to reduce this by formulating a single vaccination containing many strains and delivering it via various nanocarriers, ensuring that cellular and humoral immune responses are induced at the effector site, resulting in long-lasting protection. This leads to provide immunity against pandemic strains though memory cells. On contrary, there is still chances of influenza outbreak due to the antigenic variability. For that reason, researchers have been trying day and night to develop a universal influenza vaccine that would provide protection from all strains of influenza virus. For that reason, researchers have been working tirelessly to produce a universal influenza vaccine that would protect against all variants of influenza virus.

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