Conjugation of H1N1 Influenza peptides with PEG coated gold nanoparticles to enhance the immune response

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Submitted by

Khushpreet Kaur

Registration no. 301701014

Under the guidance of

Dr. Manoj Baranwal

(Department of Biotechnology)

Dr. Bhupendra Chudasama

(School of Physics and Material Science)

THAPAR INSTITUTE
OF ENGINEERING & TECHNOLOGY
(Deemed to be University)

DEPARTMENT OF BIOTECHNOLOGY

THAPAR INSTITUTE OF ENGINEERING & TECHNOLOGY

PATIALA-147004

JULY-2019

CERTIFICATE

This is to certify that the dissertation entitled "**Conjugation of H1N1 Influenza peptides with PEG coated gold nanoparticles to enhance the immune response''** submitted by degree of Master of Science in the subjects of Biotechnology, Thapar Institute of Engineering and Technology (TIET), Patiala is a bonafied work carried out by Khushpreet Kaur under the supervision of Dr. Manoj Baranwal, Associate Professor, Department of Biotechnology and Dr. Bhupendra Kumar Chudasama, Associate Professor, School of Physics and Material Science, Thapar Institute of Engineering and Technology (TIET), Patiala and that no part of this work has been submitted for any other degree.

The assistance and help received during the course of investigation has been fully acknowledged.

Dr. Manoj Baranwal

Associate Professor

Department of Biotechnology

Thapar Institute of Engineering and Technology

Patiala

Dr. Bhupendra Chudasama

Associate Professor

School of Physics and Material Science

Thapar Institute of Engineering and Technology

Patiala

DECLARATION

I, the undersigned, hereby declare that the work presented in the M.Sc. dissertation entitled **Conjugation of H1N1 Influenza peptides with PEG coated gold nanoparticles to enhance the immune response** has been carried out by me under the supervision and guidance of **Dr. Manoj Baranwal**, Associate Professor, Department of Biotechnology and **Dr. Bhupendra Chudasama**, Associate Professor, School of Physics and Material Science, Thapar institute of Engineering and Technology, Patiala. Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any other university or examining body in India/elsewhere.

Khushpreet Kaur

Khushpreet Kaur

301701014

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Khushpreet Kaur

Khushpreet Kaur

301701014

Abstract

H1N1 influenza is the most frequent subtype of influenza infection in people responsible for critical mortality around the world. The adequacy of antiviral medicines in treating influenza appears to be questionable due to the rise of medication safe strains. The vaccines available for the influenza virus are strain specific and require intermittent reformulation to incorporate combination of flowing flu A and B virus strains. The problem related with these vaccines is their timely and sufficient availability, if there should arise an occurrence of an unexpected outbreak. In this manner, there is an urgent requirement to develop a vaccine which can provide major protection against influenza virus worldwide. In the present study, one reported peptide (GSFVQLPELTGL) containing multiple epitopes was selected for conjugation with nanoparticles to enhance the immune response.

The immunogenic response was evaluated by repetitive stimulation of peripheral blood mononuclear cells (PBMC) with the peptide, peptide conjugated with gold nanoparticles coated with different concentrations (3.6, 8.4, 16.8, 25.2µg/mL) of poly ethylene glycol (PEG) and measure their proliferation by MTT. The 8.4µg/mL conc. of PEG coated gold nanoparticles conjugated with peptide shows the best results corresponding to other concentrations. Conservation analysis revealed that the peptide was conserved 98.4% and 95.7% in H1N1 and H5N1 respectively. Thus it is suggested that the PEG coated gold nanoparticles which are conjugated with peptides enhance immune response and can be considered in formulation of vaccine development.

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ABBREVIATIONS

AuNPs	Gold nanoparticles
CDC	Centers for disease control and prevention (USA)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FDA	Food and drug administration
HA	Hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Neuraminidase
NP	Nucleoprotein
PA	Polymerase acid
PAMP	Pathogen associated molecular patterns
PB1	Polymerase basic 1
PB2	Polymerase basic 2
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
QIV	Quadrivalent inactivated vaccine
RPMI-1640	Rosewell Park Memorial Institute -1640 medium
RPM	Revolutions per minute
TIV	Trivalent inactivated vaccine
vRNA	Viral ribonucleic acid

Chapter 1

Introduction

Influenza (commonly known as "flu") is an infectious disease that for the most part influences the upper respiratory organs (nose, throat, bronchi and occasionally lungs). The diseases caused by influenza can be mild to severe and sometimes lethal. The annual occurrence of influenza is estimated to be 5-10% in adults and 10-30% in children. The infection is transmitted effectively from individual to individual by means of respiratory droplets created when individuals sneeze, cough or spit [Morens et al., 2010]. The nearby contact (less than 1 meter) has more chances to get infected. Symptoms of this infection include; high fever, body aches, headache and severe cough, sore throat and runny nose.

Disease in humans is brought about by three types of influenza virus (Influenza A, B and C virus). These three Influenza viruses differ in their genome association, mutability, host range and pathogenecity. Among these, flu A virus is known for causing widespread disease and therefore thought to be harmful than flu B and C. Flu A virus is divided into different subtypes on the basis of two surface proteins, Hemagglutinin (H) and Neuraminidase (N). The subtypes H1N1 and H3N2 are mainly reported for human transmission and are responsible for many flu cases.

Many medications and treatments are there to treat influenza including various classes of antiviral medication. There are two classes of such drugs, adamantanes (amantadine and remantadine), and inhibitors of flu neuraminidase (oseltamivir and zanamivir). In any case, some flu infections develop protection from the antiviral medicines, minimizing the viability of treatment and also these antiviral medications are viable just when administered inside a specific time period after exposure. Likewise, antiviral medications do not confer resistance against flu.

Vaccines are there and they are the best method to control the flu. Although vaccines are available, which are successful against limited strains of flu infection, but flu keeps on mutating and gives rise to new strains. In this manner, there is an urgent requirement for the development of a universal vaccine, which could be defensive against the current and future infection strains of influenza. Out of different kinds of vaccine, peptide-based immunization is a novel

methodology since it offers appropriate choice of well-characterized antigenic epitopes with desired HLA limitations and relative simplicity of production.

Peptide based vaccines are produced as they have many advantages over the conventional whole protein vaccines. These vaccines are beneficial as they lack any deleterious sequences which may give rise to autoimmune diseases. The advantages of peptide based vaccines are: easy to synthesize with desired level of purity, devoid of any risk of autoimmunity, reversion, genetic recombination or integration. But there are some limitations of peptide based vaccines: low immune response and less stability. To overcome these limitations, adjuvants are added by which the immune response and stability of the vaccines increases. Nano based adjuvants has shown interest with some success story. Hence in the current study, one of the previous reported immunogenic peptide (GSFVQLPELTGL) of nucleoprotein (Lohia and Baranwal, 2015) was selected for conjugating with poly ethylene glycol and gold nanoparticles to enhance the immune response.

Chapter 2

Review of Literature

2.1 Influenza virus H1N1

Influenza is a highly contagious viral infection of respiratory tract which causes both endemic seasonal infections and periodic but unpredictable pandemics. The infection caused by influenza virus can vary from mild to severe and sometimes might be lethal. Individuals suffering from influenza show symptoms like cough, rhinitis, sore throat, high fever, muscle ache, headache, fatigue etc. Usually these symptoms last for 1-2 weeks, however, in some cases it can result in pneumonia, bronchitis, sinusitis, myocarditis, pericarditis, ear infections and even death. Although influenza can affect anyone, but some high risk groups have been identified which are prone to develop influenza related complications. These groups include infants (<5 years), elderly people (>65 years), pregnant women, individuals working in health care. Global annual occurrence rate of influenza is estimated to be 5–10% in adults and 20–30% in children [Mao et. al., 2012].

2.2 Structure of Influenza virus

Influenza A virus belongs to the *Orthomyxoviridae* family. In view of the antigenicity of their hemagglutinin (HA) and neuraminidase (NA) particles, flu A is classified into 16 HA subtypes (H1-H16) and 11NA subtypes (N1-N11) mentioned in CDC (Centers for Disease Control and Prevention) report. Influenza A infections contain a genome made out of eight sections of single-stranded, negative-sense RNA that each encodes a couple of proteins. Influenza virus contains haemagglutinin (HA) and neuraminidase (NA) proteins. HA enables the infection to contaminate cells through cooperation with sialic-corrosive deposits on receptors and NA is a receptor-destroying enzyme which spreads the infection by enabling the virus to escape from infected cells [Gamblin et. al., 2010].

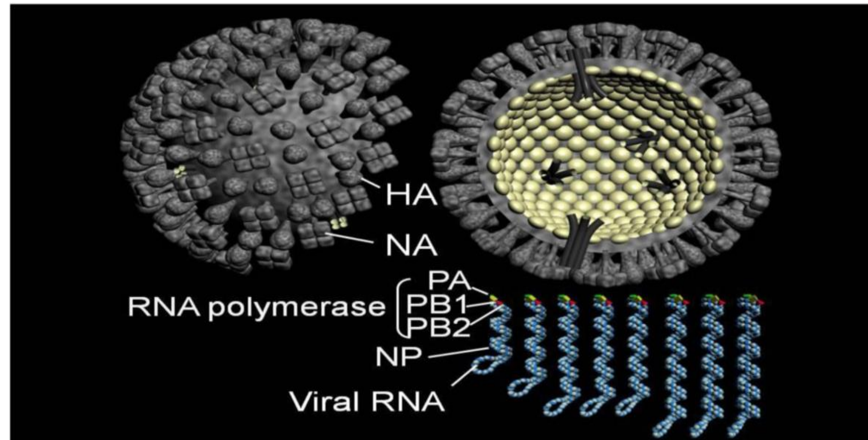


Figure 2.1: Schematic diagram of Influenza A viruses

(Source: https://www.nature.com)

Influenza virus secures a lipid layer during development by growing through the host cell membrane [Laver et. al., 1983]. The coming about viral film contains the two virally coded basic layer glycoproteins, haemagglutinin and neuraminidase as shown in Figure 2.1 [Wilson et. al., 2012]. The influenza A viral heterotrimeric polymerase complex (PA, PB1, PB2) is known to be engaged with numerous parts of viral replication and to cooperate with host factors, in this manner having a job in host particularity [Jeffery et. al., 2005]. In humans and domestic animals, influenza A viruses cause annual outbreaks.

Influenza virus is subjected to small amount of antigenic drift and prime amount of antigenic shift which permit the virus to control the immune response of mammalian and avian species [Zambon, 1999].

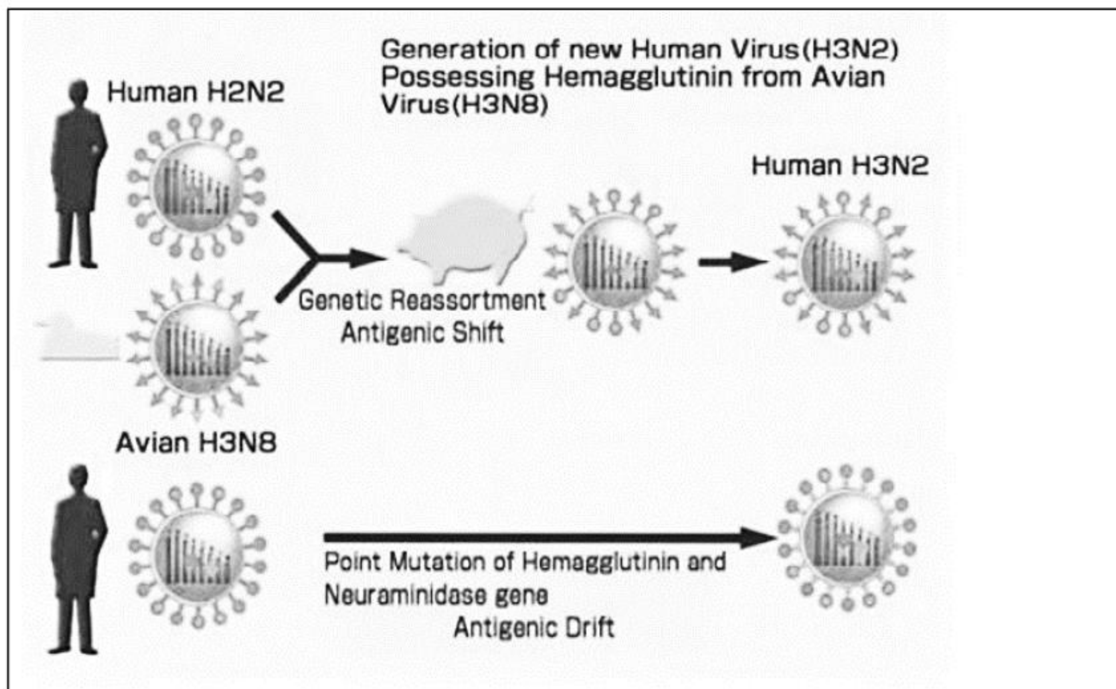


Figure 2.2: Antigenic shift and antigenic drift

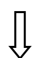
(Source: https://www.ncpresbytery.org)

Antigenic drift includes point transformation, each season of infection reproduces, and prompting the fast spread of new popular strain among the population coursed a long time before. Antigenic drift happens in all strains of influenza A and B infections though antigenic shift is recognized distinctly if there is arise in occurrence of influenza A infection, which makes them most hazardous among all the flu infections. Along with this influenza A infections are regular just like pandemic outbreaks as shown in Figure 2.2. Antigenic shift is the procedure of hereditary reassortment (blending of hereditary material between various viral strains), offering another infections which are never been available in human course [Zambon, 1999].

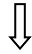
2.3 Lifecycle of Influenza

The life cycle of influenza virus is divided in five stages [Samji et. al., 2009]

Entrance of infectious molecule into the host cell.

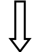


Entrance of infectious Ribonucleoprotein (RNP) in the nucleus.



Transcription and replication of the viral genome.

Export of vRNPs from the nucleus.



Assembly and budding at the host cell plasma membrane.

The sialic acid, which is found outside of the host cells membrane. After this binding, receptor-mediated endocytosis happens and the infection enters the host cell in an endosome. The endosome has a low pH, which triggers the combination of the viral and endosomal layers as shown in Figure 2.3. This low pH makes a conformational change in HA directing the development of fusion peptide. This fusion peptide embeds itself into the endosomal film, bringing both the viral and endosomal layers into contact with one another [Huang et. al., 2003]. The acidic condition of the endosome opens up the M2 particle channel. Opening the M2 particle channels ferments the viral center. This acidic condition in the virion discharges the vRNP from M1 with the end goal that vRNP is allowed to enter the host cell's cytoplasm [Pinto et. al., 2006]. NP, PA, PB1 and PB2 are the viral proteins which makes up the vRNP. These proteins have nuclear localization signals (NLSs) which can bind to the cellular nuclear machinery and in this way, enters the nucleus [Kielian et. al., 1990].

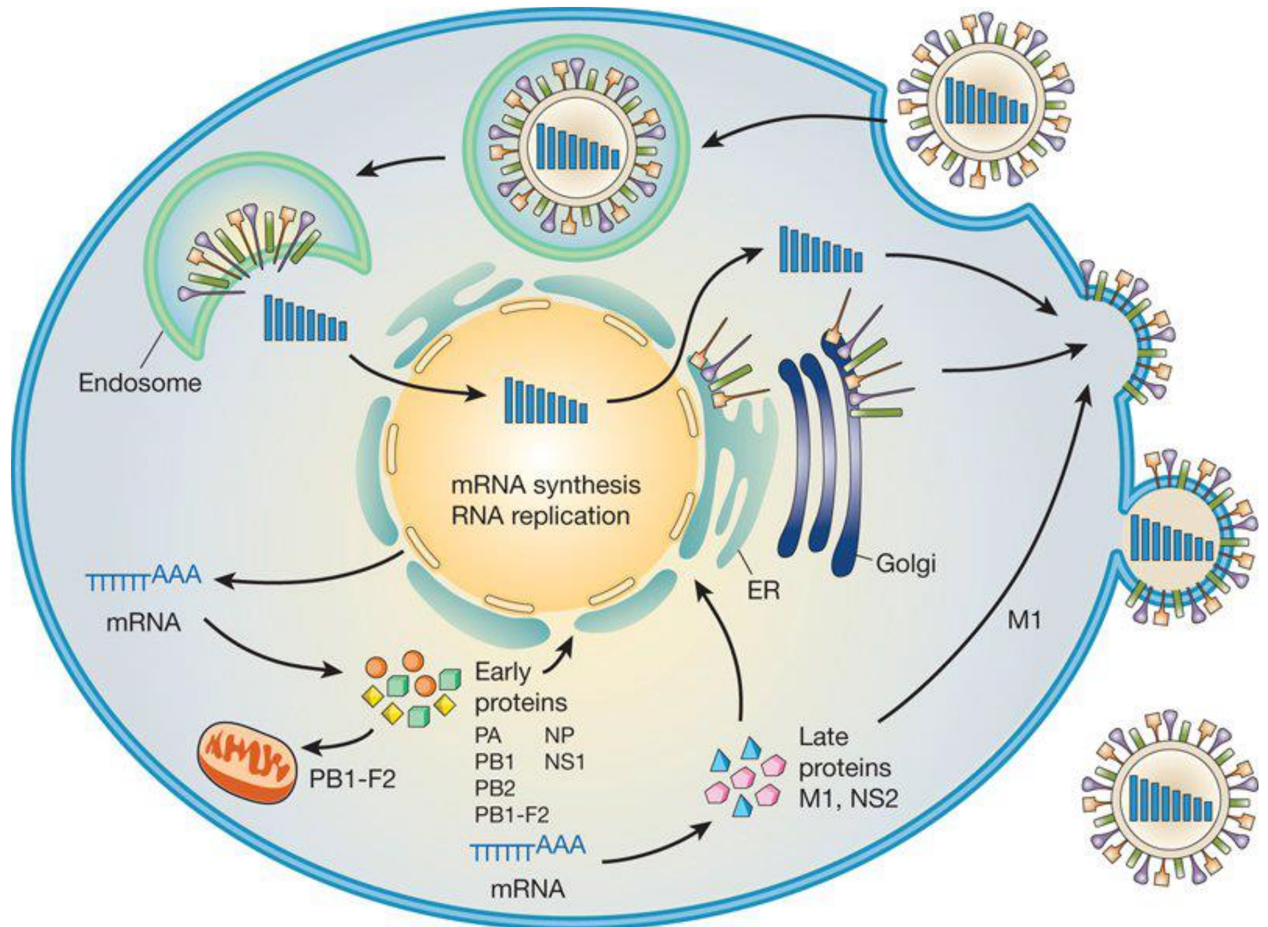


Figure 2.3: Life Cycle of Influenza Virus

(Source: https://synapse.koreamed.org)

The viral polymerase complexes interprets and duplicates the viral RNAs in the nucleus. Flu viral genome is negative sense RNA which is first changed over into a positive sense RNA. Positive sense RNA is currently utilized as a template for RNA replication which is done by viral protein PA. After replication viral RNA is transcribed to mRNA of various viral proteins which is completed by PB1 protein. Viral mRNA does not have 5´ cap so PB2 works as endonuclease and divides 5´ methylated caps of cell mRNA (10 – 15 nucleotides). This cell capped RNA piece is utilized as a primer for viral transcription [Li et. al., 2001]. Viral mRNAs move to cytoplasm where they are interpreted. HA, NA and M2 proteins are moved to cell film while different proteins like NP, M1, NS1 and NEP (nuclear export protein) move to the nucleus where they tied to the viral RNA shaping Ribonucleoprotein complex (RNP). This RNP relocates into the cytoplasm in a NEP-dependent process and in the end collaborate by means of M1 with an area

of the cell layer, where HA, NA and M2 have been embedded. At that point the recently incorporated virions bud from tainted cell. NA destroys the sialic acid moiety of cell receptors, in this way discharging the progeny virions [Bao et. al., 2008].

2.4 Neuraminidase (NA) protein

Neuraminidase is a protein which is present on the outside of influenza protein. NA has an important function of removing sialic acid from glycoproteins. For the release of viral progeny, the expulsion of sialic acid moiety from the surface protein of the host cell just as newly formed virions is very essential. One of the findings suggested that removal of sialic acid from the cilia for respiratory tract, mucine and glycocalyx but NA limits the access of virus to the other target cells [Matrosovich et. al., 2004]. The sialic acid is present on the cellular surface of proteins and on the viral glycoproteins, sialic acid is present; it is a cell receptor where via the HA protein, influenza virus attaches.

2.5 Prevention and control of Influenza

2.5.1 Vaccine

The most effective way to stop influenza infection is vaccination [Woodland, 2016]. For controlling infectious diseases, the most cost-effective strategy is Vaccination. Edward Jenner is the first who documented the case of vaccination in 1976 against small pox. Vaccination has been instrumental in diminishing fatalities related with dangerous illnesses including polio, measles, and diphtheria [Preety et. al., 2014].

Existing flu vaccines are powerful against infection yet require occasional reformulation for frequent mutations (antigenic drift). Advancement has been made in the previous vaccine formulations to improve the immunity against seasonal pandemic.

2.5.1.1 Licensed influenza vaccines

The extensively used vaccine for seasonal influenza is trivalent inactivated vaccine (TIV). It is composed of two influenza A subtypes (H1N1 and H3N2), present in the human circulation and one influenza B virus. TIV protects against flu primarily by evoking anti-HA antibody production. Flu PR8 strain (A/Puerto Rico/8/34) or reassortant infection which has been adjusted

to develop in embryonated chicken eggs with high development capacity is utilized for inactivated flu vaccine production. This vaccine comes in three distinct groups: inactivated whole virus, split and subunit. An inactivated whole virus vaccine is a production of purified virus, which is synthetically inactivated by formalin or β-propiolactone treatment. It was first endorsed for use in Unites States in 1975. Split preparation is produced by nonionic detergent interceded disruption of flu infection to expel the lipid envelop. Subunit preparation needs extra purification of HA. Split and subunit groups of TIV are preferred over whole virus vaccines as a result of their less reactogenicity in primed population. Quadrivalent inactivated vaccine (QIV) against flu consolidates circulating strains of H1N1 and H3N2 subtypes of flu A infection and two strains of flu B infection [Bekkat et. al., 2016]. To avoid the contradiction which occurs to Influenza B strains, it was mainly designed. FDA has endorsed the manufacturing of a quadrivalent split vaccine named Fluarix (GlaxoSmithKline Biologics) [Bekkat et. al., 2016].

Live attenuated influenza virus (LAIV) vaccines against influenza are available as intranasal spray (FluMist). Strains (master donor virus) of LAIV are cold adjusted, temperature sensitive and attenuated to forecast illness by methods of arrangement of mutations in the internal protein coding gene segments. LIAV is a reassortant infection antibody made up of wild kind of surface glycoproteins and internal proteins of master strain. The usage of this vaccine has not approved for adults (>50 years) [Hoft et. al., 2011].

2.5.1.2. Whole virus vaccines

GammaFlu^{TM} is a whole virus broad spectrum vaccine developed by Gamma vaccine (Australia) [Nichol et. al., 2006]. It is a formulation of gamma irradiated whole flu virus, in whole the hereditary material of the infection is wrecked (non-replicating), leaving all the surface and internal protein intact [Berlanda et. al., 2015]. This methodology has been shown to produce powerful immune response against homologous and heterosubtypic challenges in mice. This cross defensive immunity was observed to be mainly intervened by cytotoxic T cells in quality knockout mice [Furuya et. al., 2010]. Conserved sequences which are shared by different flu viruses can be used as vaccine antigens, for the development of universal flu vaccines [Wenqian et. al., 2017].

Subunit vaccines are the new generation vaccines, including recombinant proteins, peptides and DNA. Their main limitations are weak immunogenicity and short-term immune response.

2.5.2. Peptide based vaccines

Peptide based vaccine production has picked up many advantages in recent past over the conventional whole protein vaccine. Peptide based vaccines have gained advantages because of their stability, cost effectiveness and easy manufacturing (Ovsyannikova, 2007). Peptide vaccines are beneficial as they are lack of deleterious sequences which may give rise to autoimmune diseases [Arnon et. al., 2006]. The adjuvants are added with peptide based vaccines to enhance stability and immune response. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is mainly an adjuvant which has been used with peptide based cancer vaccines and given the good results for raising the immune response [Clive et. al., 2010].

Hence, it is obvious that several approaches leading to formulation of peptide based influenza vaccines are under huge research and development globally. Peptide based vaccines are an attractive alternative strategy that relies on usage of short peptide fragments. It can be engineered for the induction of highly targeted immune responses.

2.5.2.1. Advantages of peptide based vaccine

- Devoid of any risk of autoimmunity, reversion, genetic recombination or integration.
- Peptide vaccines are non allergic.
- Ease of large scale production, modification and transportation.
- Ease to synthesize with desired purity level.

2.5.2.2. Limitations of Peptide based vaccines

- Low immune response
- Less stable

To overcome these limitations, adjuvants are added for enhancement of immune response and stability of the peptide vaccines.

2.6 Adjuvant

An adjuvant is a substance that modulates the immune response to an antigen to improve them. Adjuvants are added to a vaccine to increase the immune response to form more antibodies and longer-lasting immunity, thus decreasing the amount of antigen needed.

New age vaccine, especially those which are dependent on recombinant proteins, peptide and DNA, are probably going to be less reactive than traditional immunizations, and on the other hand are less immunogenic. Hence, there is an urgent requirement for the advancement of better than ever vaccine adjuvants [Krammer et. al., 2015]. Adjuvants can be extensively distinguished into two classes, on the basis of their mode of action; antibody delivery systems and immunostimulatory adjuvants. These immunostimulatory adjuvants prevalently represent pathogen associated molecular patterns (PAMP). The revelation of increasingly intense adjuvants may permit the improvement of vaccines against irresistible agents, for example HIV which don´t normally inspire defensive resistance. New adjuvants may enable immunizations to be delivered mucosally [Derek et. al., 2001].

2.7 Nanotechnology and Biotechnology

Nanotechnology uses the properties of objects that work as a unit inside the general size range of 1 to 1,000 nanometers, which is on a similar scale concerning numerous organic structures, for example, antigens, receptors, subcellular parts of the invulnerable framework and microorganisms. The building of nanoscale mixes by the change of properties, for example, nanoparticle measure, shape, charge, porosity, surface zone and hydrophobicity holds incredible guarantee for the advancement of resistant reaction modulators and immunizations.

The improvement of the immune response by nanoparticles can be accomplished through inborn safe potentiation or by the upgraded delivery of antigens. Nanoparticles enact the inborn insusceptible reaction by means of Toll-like receptors and the repetitive presentation of antigens, and good immunization carriers.

Nanotechnology will keep on giving amazing bits of knowledge into the idea of the resistant reaction. The utilization of nanotechnology to immunology may likewise affect new techniques to prevent or to treat human infections [Douglas et. al., 2013]. Gold nanoparticles are

used to convey an innate immune activator, producing a restricted therapeutic reaction. [Krishnan et. al., 2010]. Gold (in the form of nanoparticles), is used in medicines because of its therapeutic effects on many diseases. Gold also plays a vital role in vaccine field as an adjuvant, minimizing the toxicity, enhancing the stability and immunogenic activity [Sonia et al., 2017]. Nanoparticles have given a unique platform for the target-specific drug delivery agents [Partha et. al., 2008]. There are many unique properties of gold nanoparticles such as; inert and non-toxic [Partha et. al., 2008].

When gold nanoparticles are conjugated with peptide or any other functionalized groups, they can be used for therapeutic applications. The physical interaction between gold nanoparticles and peptides depends on: i) Ionic interaction between positively charged peptide and negatively charged gold nanoparticles. ii) Hydrophobic attraction between both peptide and gold nanoparticles. iii) Binding between gold electrons and amino acids of peptide [Hamed et.al., 2016].

The rise of co-contaminations and the advancement of medication safe pathogens limit the utility of current treatments against infections, and developing nations specifically are confronting an extraordinary challenge in fighting infectious disease. Besides, any inability to control the spread of irresistible sickness would likewise speak to a risk to developed nations. Ongoing advancements in nanotechnology enable us to address this issue at two dimensions: diagnostics and treatment. Counteractive action of the spread of irresistible pathogens requires fast and precise identification of the irresistible agents for appropriate treatment. Legitimate and compelling treatment spares the patient, yet in addition anticipates the spread of the pathogens. Specific nanoparticle vehicles created to embody therapeutic agents and convey them to an objective site represent a promising system to support invulnerable reactions for vaccination and boost the adequacy of medications for treatment.

The Gold nanoparticles (AuNPs) are successful vaccine adjuvants and upgrade the immune response by means of cytokine pathways based upon different sizes. [Kenichi et. al., 2013].

The coating of Polyethylene glycol (PEG) and bioconjugates (gold nanoparticles with peptide), are highly stable and it is characterized by the nanoparticles size and monolayer

composition [Mathew et. al., 2007]. Poly ethylene glycol (PEG) has been among the most prominent candidates used to manufacture drug delivery system and tissue engineering applications. PEG is biocompatible and biodegradable, shows a wide scope of disintegration times, has tunable mechanical properties and above all, is a FDA affirmed polymer [Hirenkumar et. al., 2011]. Specifically, PEG has been broadly considered for the advancement of gadgets for controlled delivery of little atom drugs, proteins and different macromolecules in commercial use and in look into. [Hirenkumar et. al., 2011].

One study revealed that macrophages were unfit to perceive gold nanoparticle peptide conjugates, while peptides or nanoparticles alone were not perceived [Ester et. al., 2019]. Antibodies are the best development which is being used for the different diseases when any foreign antigen enters the body. An immune response is created during the entrance of an antigen which is predominantly less immunogenic. Because of the less immunogenic response by certain immunizations, the need of adjuvant has been raised. The most normally utilized adjuvants are the aluminium adjuvants which are utilized for the human vaccination. But because of low effectiveness, low stability, tolerability, and poisonous quality polymeric carrier for adjuvant are utilized. So there is a requirement for the improvement of new adjuvants so they can profit the field of immunology. In one of the study, polymeric carrier utilizing sodium alginate/Chitosan beads were shaped in which the peptide was encapsulated. Raw beads communication examine, MTT measures were performed so as to describe the peptide loaded beads. In this way, the impact of Chitosan/sodium alginate globules (with and without peptide) on peripheral blood mononuclear cells (PBMC) and RAW 264.7 cell lines were optimized. Sodium alginate dots were found to have no impact on PBMC as the absorbance worth was discovered like that for cells. In peptide embodied sodium alginate/Chitosan beads PBMC associated and proliferated more proficiently within the sight of peptide typified in Chitosan/sodium alginate beads as the absorbance worth was observed to be more than cells just and peptide treated cells. Definitively, Chitosan/sodium alginate globules can be used as a promising delivery vehicle in the wake of consolidating peptide based nanoadjuvants for upgrading the immune response [Khyati et. al., 2017].

Many nanoparticles like gold, carbon, polymers, and liposomes have the ability to unite with peptides to enhance the immune responses and stability. All these nanoparticles have the

abiltity to enter inside the cells by endocytosis mechanism because of their small size [Treuel et. al., 2013].

Here, we describe an assortment of nanotechnology with biotechnology for use in applications, for example, safe reaction balance, drug delivery, diagnostics and treatment, which are particularly required in developing nations [Qasim et. al., 2014].

Chapter 3

Objectives

- Synthesis and characterization of hybrid nanoparticles consisting of Gold and Polyethylene glycol (PEG).

- Conjugation of H1N1 peptides with PEG coated gold nanoparticles.

- Evaluation of immunogenic response of nano-conjugated peptides in peripheral blood mononuclear cells.

Chapter 4

Materials and Methods

Materials used:

Table:1. List of chemicals and reagents

S.No.	Reagents/Chemical	Company
1.	Ascorbic Acid	Merck Specialties Private Limited
2.	Bovine Serum Albumin	Sigma Aldrich
3.	Concavalin A	Sigma Aldrich
4.	Foetal bovine serum	Gibco@Life Technologies, USA
5.	HEPES buffer	Sigma Aldrich
6.	$HAuCl_4$	Sigma Aldrich
7.	MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)	Sigma Aldrich
8.	Pluronic F-127	Sigma Aldrich
9.	Streptomycin	Sigma Aldrich
10.	Silver nitrate	Sigma Aldrich
11.	Trypan blue	Himedia
12.	Tween-20	Sigma Aldrich
13.	Polyethylene glycol	Rankem
14.	Histopaque	Himedia
15.	Phosphate buffer saline	Sigma Aldrich
16.	DMSO (Dimethyl sulfoxide)	Himedia

Methodology

4.1 Synthesis of Gold Nanaoparticles (Au NPs):

Growth solution of Gold nanoparticles (AuNPs) was prepared by mixing 5mL of 1.0 mM $HAuCl_4$ (Chloroauric acid) solution with 5mL of 100 mM aqueous solution of pluronic F-127. To this homogenous mixture of $HAuCl_4$ and pluronic F-127, 0.2 mL $AgNO_3$ was added. 45 µL of 0.1 M ascorbic acid was added dropwise to the solution, until the colour changes. The solution was kept undisturbed for 24 h at room temperature. AuNPs were centrifuged at 10,000 rpm for 10 min. Pellet was discarded. The colloid was preserved for further processing.

4.2 Coating of AuNPs with PEG (Polyethylene glycol):

Coating of AuNPs is done with four different concentrations of PEG (3.6, 8.4, 16.8, and 25.2 µg/mL). PEG solution of each concentration was prepared. As-synthesized gold nanoparticles were divided in four equal volumes and 5 mL of each PEG concentration solutions were added respectively. The solutions were stirred continuously for 4 h. PEG coated AuNPs were preserved for further processing.

4.3 Conjugation of PEG coated AuNPs with Peptide:

500 µl of PEG coated AuNPs solution and 500 µL of neuraminidase protein peptide solutions were mixed properly and stirred continuously for 4 h on magnetic stirrer at room temperature. Peptide conjugated AuNPs were preserved at room temperature for further measurements.

4.4 Isolation of peripheral blood mononuclear cells (PBMC):

Five ml blood was drawn from healthy volunteers by trained technicians of Rajindra Nitin Hospital, Patiala (India) in blood collection EDTA coated tubes (BD Vacutainer® Tubes). The informed consent was taken from volunteers and the study was approved by institutional ethical committee. Peripheral Blood Mononuclear Cells (PBMC) were isolated by ficoll density gradient method (Kumar et al., 2006). Five ml of blood was carefully layered on equal volume of Histopaque and centrifuged at 400g for 30 minutes at 25°C in swinging bucket rotor. This centrifugation technique fractionates blood into

plasma, peripheral blood mononuclear cells (PBMC) and red blood cells. After centrifugation, the upper plasma layer was discarded using micropipette and opaque interface (buffy coat) containing PBMC was collected in sterile 15ml centrifuge tube. The cells were washed with 6-8ml of 1X phosphate buffer saline (PBS) in the same swinging bucket rotor at 330g for 12 minutes at 25°C. At last the cell pellet was re-suspended in 1ml of complete media (RPMI-1640 supplemented with 10% of foetal bovine serum, 100µg/ml streptomycin, 100 IU/ml penicillin and 10mM HEPES).

4.5 PBMC stimulation assay

In order to test the immunogenicity of peptide, isolated PBMCs were stimulated with the peptide and peptide conjugated nanoparticles. In a flat bottom 24 well cell culture plate, 2×10^6 cells were stimulated with the peptide and other sample solutions in the presence of complete RPMI-1640 media in total volume of 200µl. Unstimulated cells served as control. The cultured plate was incubated at 5% CO_2 at 37°C in a humidified incubator. The cells were restimulated with peptide and other conjugated peptide samples on 4th day and kept at same incubation conditions. On 5th day 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was performed to estimate the proliferation of peptide stimulated cells as compared to the controls. 120µl of solution was taken and stored in separate eppendorfs from each well. 20µl of MTT (5mg/ml) was added to each well and incubated for 4 hours. 100µL of Dimethyl sulfoxide (DMSO) was added to each well to dissolve the crystals. Absorbance was measured at 570/630nm in multi-well plate reader.

4.6 Characterization of AuNPs:

As synthesized and coated AuNPs were characterized by UV-Visible spectroscopy, Dynamic Light Scattering (DLS) and FTIR spectroscopy. UV-Visible spectra was recorded on Shimadzu 2600 spectrophotometer from 300-800 nm at room temperature, DLS measurements were performed on Brookhaven 90 plus particle size analyzer. FTIR spectra was recorded on Agilent Cary-600 Series.

4.7. Conservancy analysis

4.7.1. Sequence Retrieval

The sequence of neuraminidase protein of H1N1, H2N2, H3N2, H5N1 and H7N9 were obtained from Influenza Research Database (https://www.fludb.org). The duplicate sequences were removed by using the option given in the database and the full length sequences were downloaded.

4.7.2. Multiple Sequence alignment (MSA)

By using Clustal Omega this multiple sequence alignment was done [Sievers et.al.,2011]. Clustal Omega is a good method of multiple sequence alignment which can fastly align the number of protein sequences and give accurate alignments.

4.7.3. Identification of conserved Regions

Conservancy analysis was carried out to find the conserved regions present in the Neuraminidase Protein of different subtypes of influenza virus. AVANA tool (Antigen Variability ANAlyzer) was used for the conservancy analysis.

To measure the variability in protein sequence alignments, AVANA tool uses the information entropy [Miotto et. al., 2008].

The multiple sequence alignment results of Clustal Omega (FASTA format) was used as an input for AVANA software. The conserved peptide region was searched in the alignments. The percentage of conservancy will appear.

Chapter 5

Results

5.1. Synthesis of AuNPs:

Synthesis of AuNPs was monitored by UV-visible spectrophotometer. The range of gold particles lies between 400-700nm in visible spectrum. UV-Visible spectra of as-synthesized gold nanoparticles is shown in Figure 5.1.1. A single SPR (Surface Plasmon Resonance) peak confined around 560 nm was observed in the UV-Visible spectra. This plasmonic peak in the visible region confirms the formation of AuNPs.

In Figure 5.1.2. UV-Visible spectra of PEG coated AuNPs is shown. No spectral shift in the plasmon bend is observed in samples which are coated with different concentrations of PEG. This indicates that PEG coating do matter

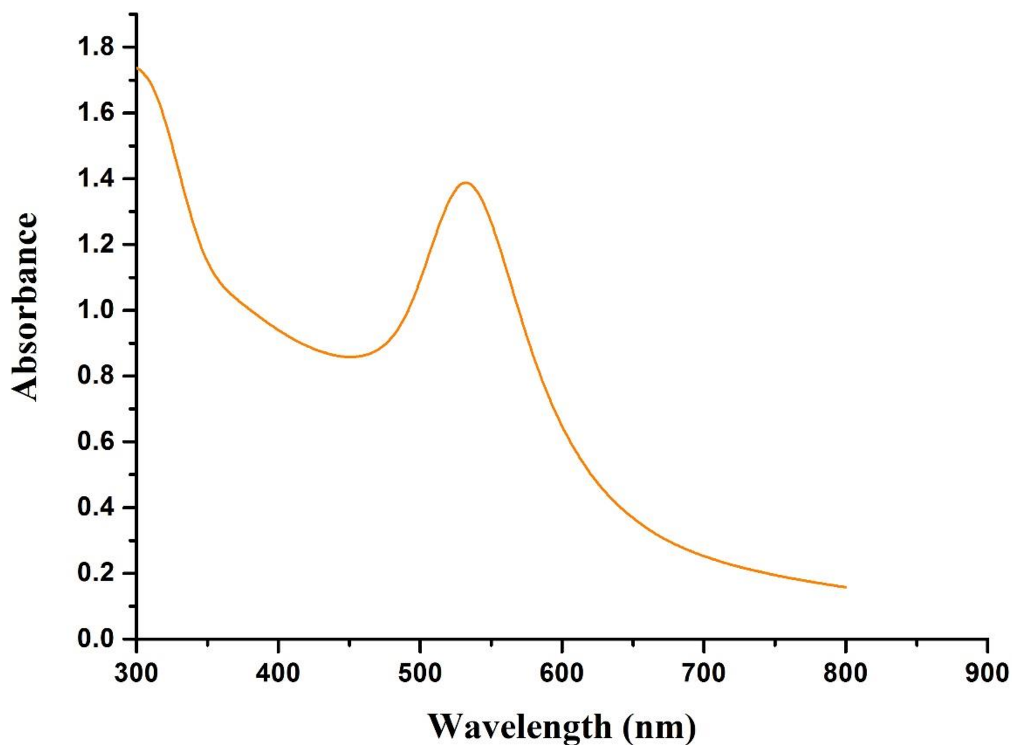


Figure 5.1.1. UV-Visible spectra of as-synthesized AuNPs

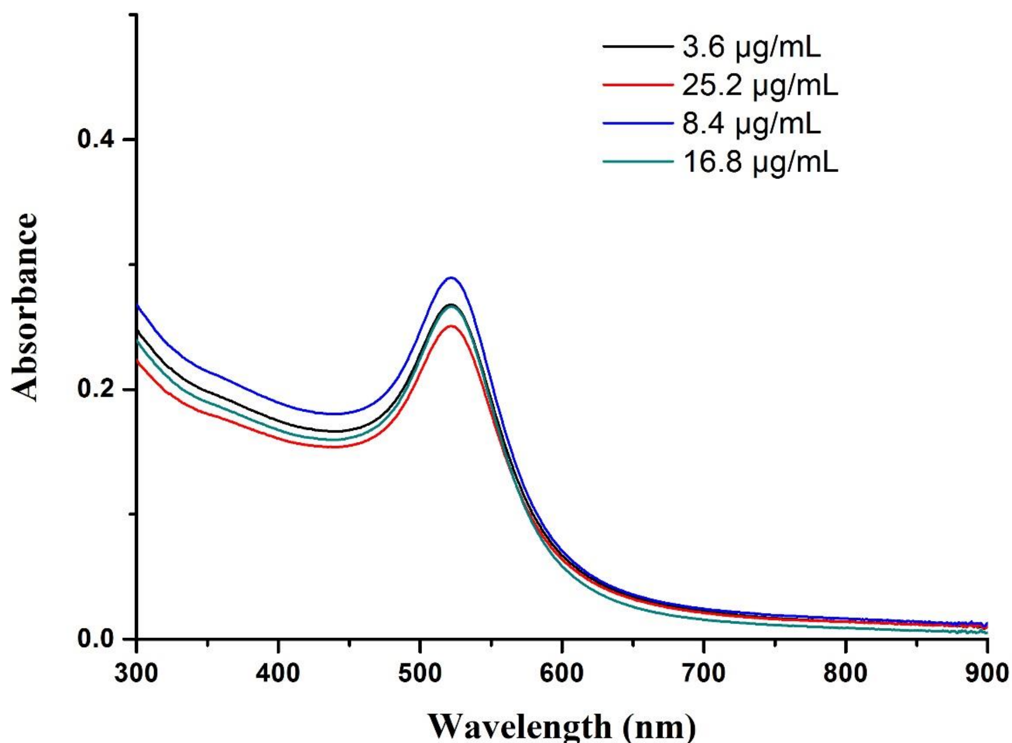


Figure 5.1.2. UV-Visible spectra of AuNPs coated with different concentrations of PEG

5.2. Dynamic Light Scattering (DLS)

This technique is used to determine the hydrodynamic size of small particles in solution.

Figure 5.2.1. shows the size distribution of as-synthesized AuNPs. Mean hydrodynamic size is 22 nm.

Figure 5.2.2. shows the size distribution of 3.6µg/mL PEG coated AuNPs. Mean hydrodynamic size is 29 nm.

Figure 5.2.3. shows the size distribution of 8.4µg/mL PEG coated AuNPs. Mean hydrodynamic size is 24 nm.

Figure 5.2.4. shows the size distribution of 16.8µg/mL PEG coated AuNPs. Mean hydrodynamic size is 29 nm.

Figure 5.2.5. shows the size distribution of 25.2µg/mL PEG coated AuNPs. Mean hydrodynamic size is 27 nm.

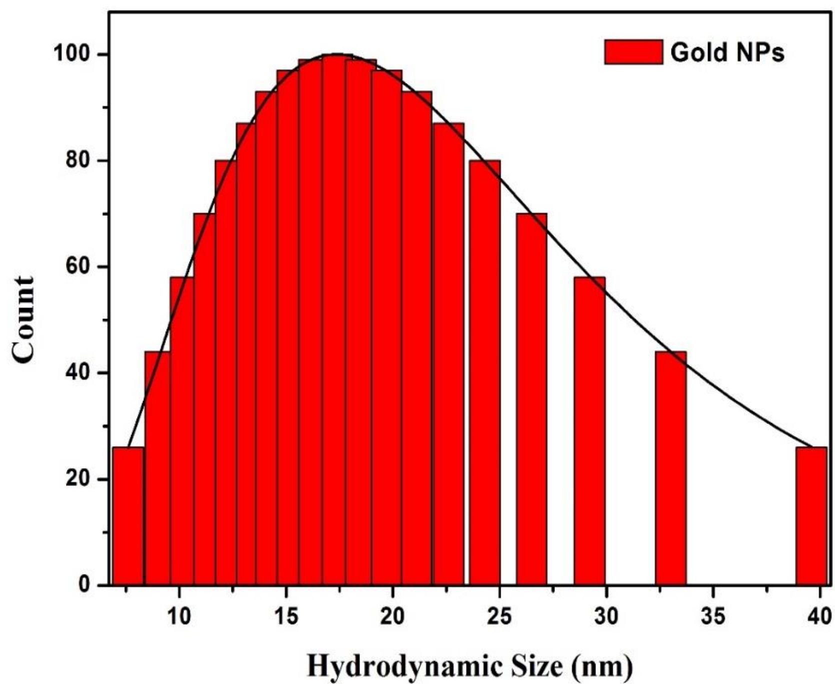


Figure 5.2.1. Particle size distribution of as-synthesized AuNPs

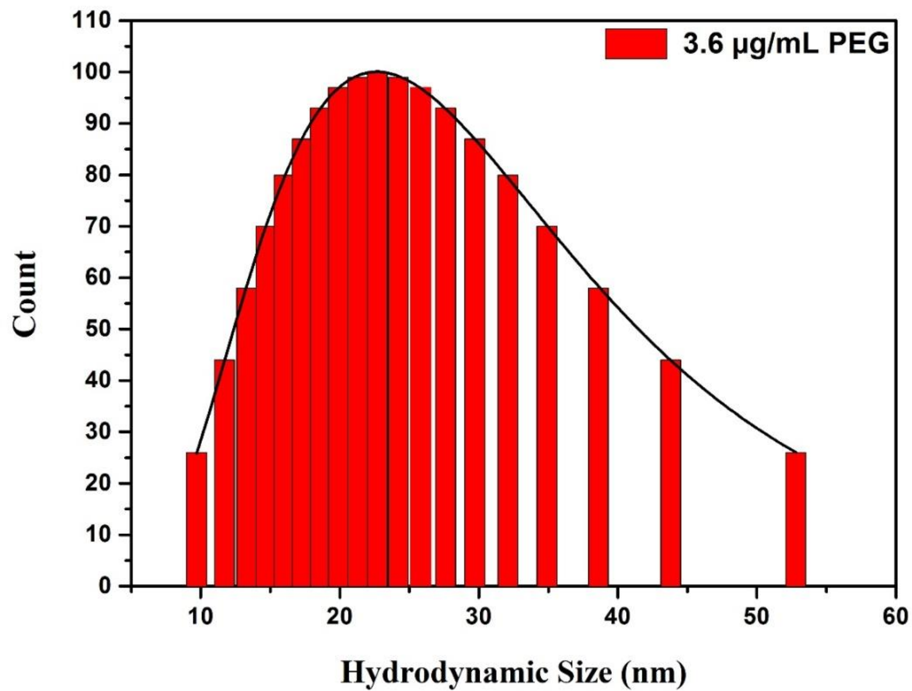


Figure 5.2.2. Particle size distribution of PEG (3.6µg/mL) coated AuNPs

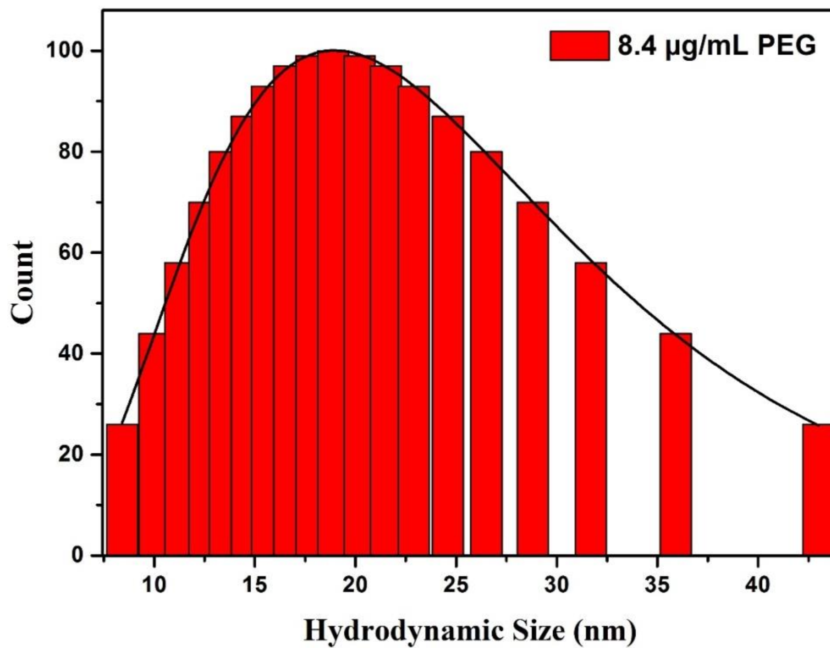


Figure 5.2.3. Particle size distribution of PEG (8.4µg/mL) coated AuNPs

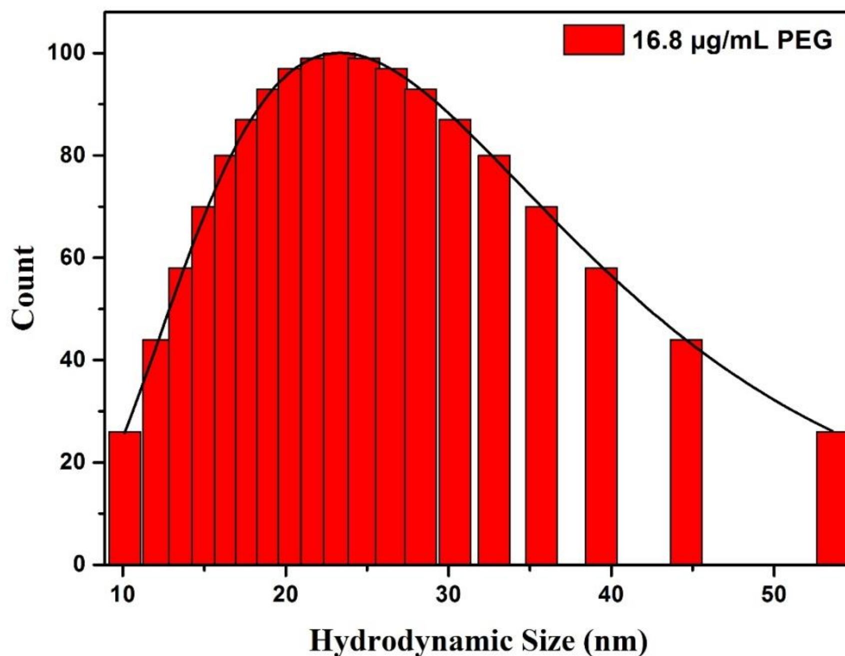


Figure 5.2.4. Particle size distribution of PEG (16.8µg/mL) coated AuNPs

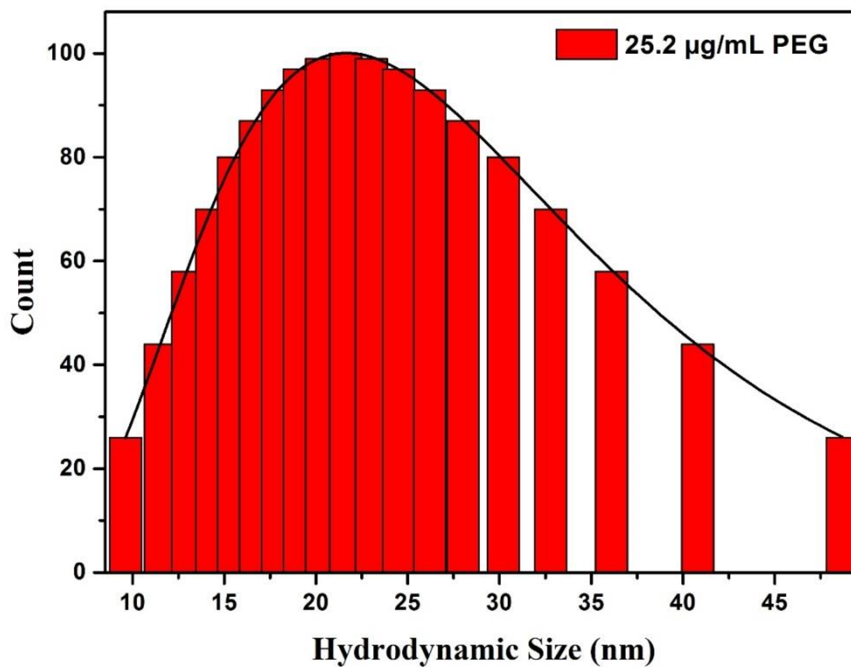


Figure 5.2.5. Particle size distribution of PEG (25.2µg/mL) coated AuNPs

5.3. Fourier-transform infrared spectroscopy (FTIR)

The conjugation of the nanoparticles with the neuraminidase peptide was determined by this technique.

Figure 5.3.1. and Figure 5.3.3. are the pure neuraminidase peptides N5 and N6 respectively.

Figure 5.3.2. and Figure 5.3.4. are the peptides conjugated with gold nanoparticles.

Characteristic peaks of peptide are observed in all the samples indicating the physical adsorption of peptide over PEG-coated AuNPs.

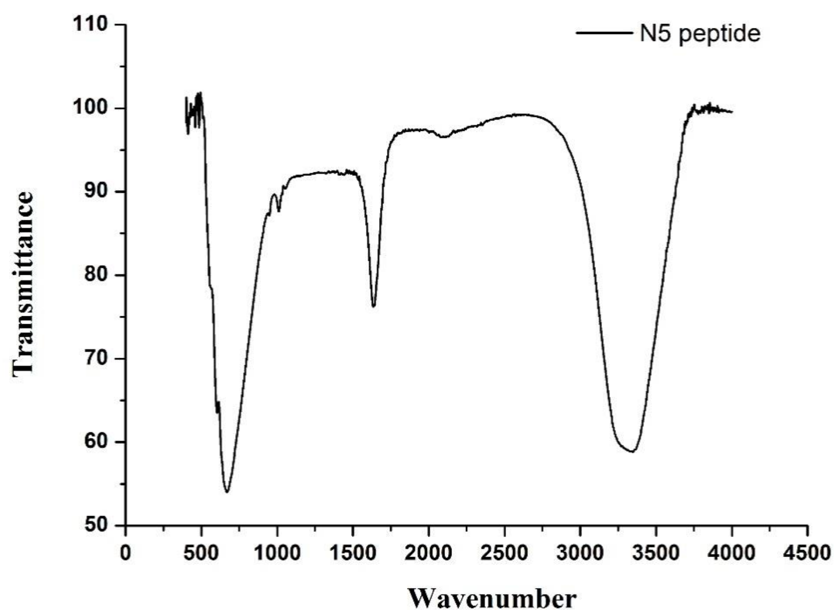


Figure 5.3.1. FTIR spectra of pure neuraminidase protein peptide (N5)

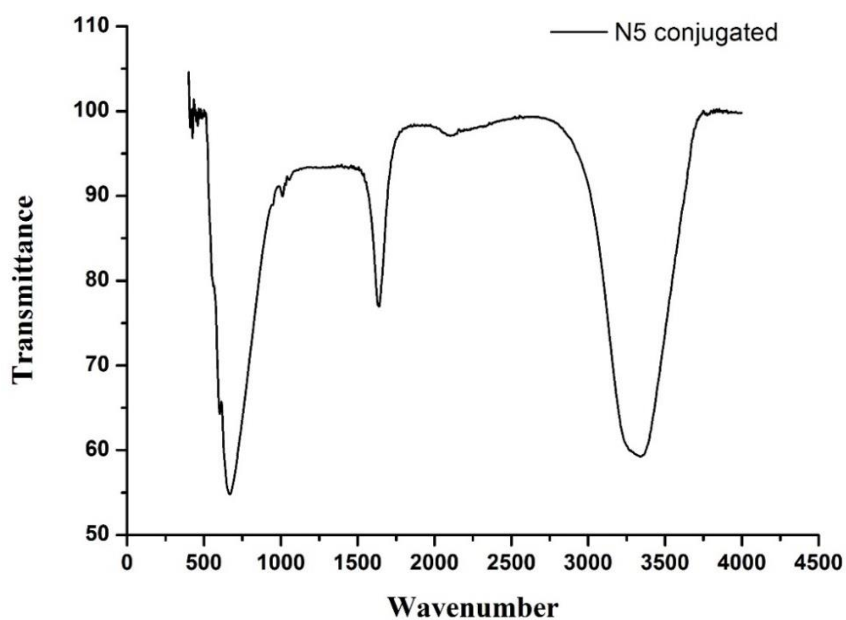


Figure 5.3.2. FTIR spectra of N5 peptide conjugated with 8.4µg/mL PEG conc. AuNPs

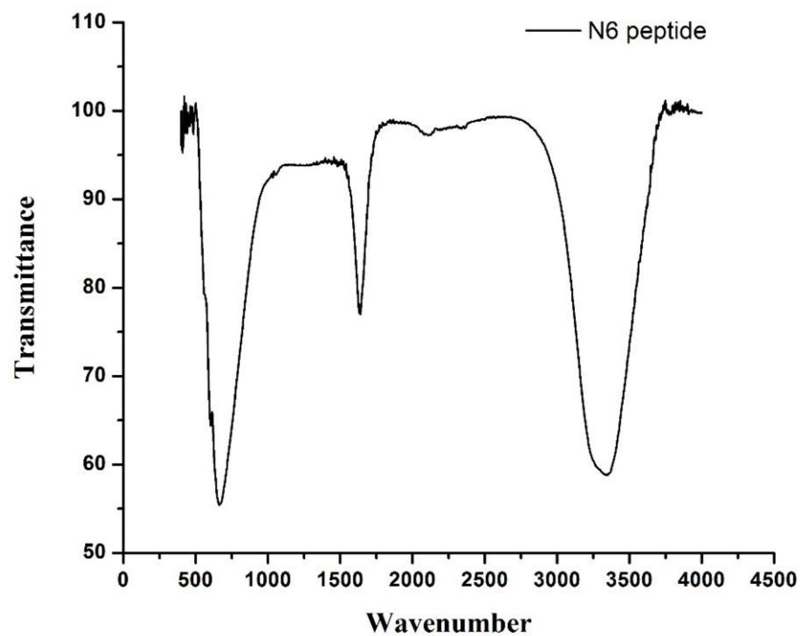


Figure 5.3.3. FTIR spectra of pure neuraminidase protein peptide N6

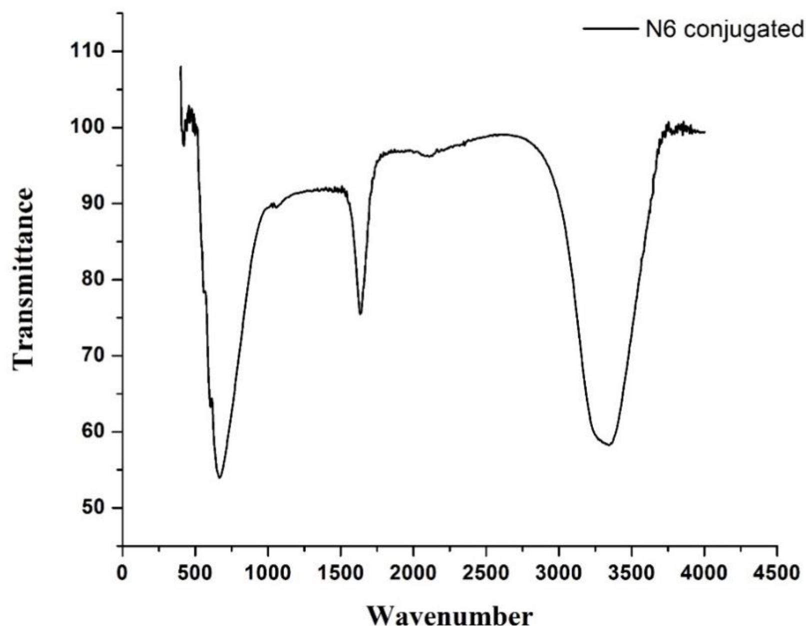


Figure 5.3.4. FTIR spectra of N6 peptide conjugated with AuNPs

5.4. Neuraminidase peptide induced PBMC proliferation after conjugating with polyethylene glycol (PEG) coated gold nanoparticles

MTT assay was carried out to check the effect of NA peptide induced proliferation of PBMC when peptide is conjugated with polyethylene glycol (PEG) coated gold nanoparticles. Four different concentrations (3.6, 8.4, 16.8 and 25.2µg/mL) of PEG was used for coating of the gold nanoparticles and then conjugating with NA peptides. The enhanced proliferation was observed in all four concentrations of PEG coated gold nanoparticles conjugated with NA peptides (Figure 5.4.1). 8.4µg/mL was found to be the best in all concentrations hence this concentration was used for the repetition of experiments. 8.4µg/mL has shown enhanced proliferation in all the experiments as shown in Figure 5.4.2. Concavalin A was used as positive control and shown stimulation index more than 3.8.

Concavalin A (ConA) (as a positive control), Peptide (P_N5), Four Polyethylene glycol (PEG) coated gold nanoparticles conjugated with neuraminidase peptide (P_N5) were tested for their immunogenic potential in PMC sample and stimulation index was calculated to analyse the

response of these samples. PCP2 was found to be best among all the four nanoparticles conjugated samples (Figure 5.4.1.).

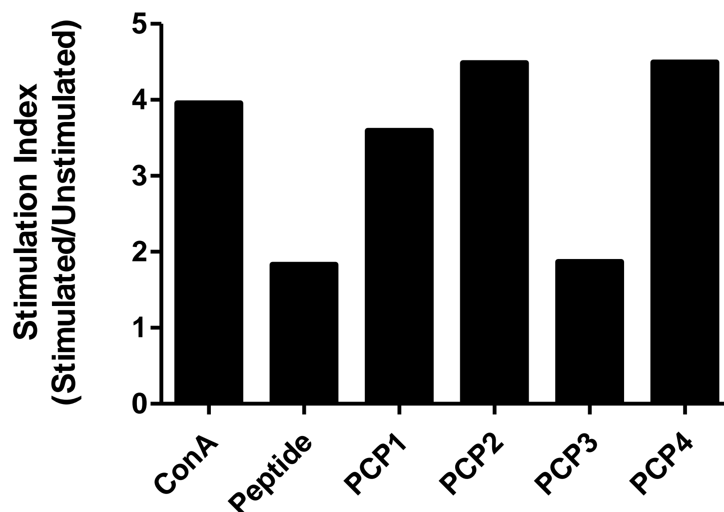


Figure 5.4.1. Neuraminidase peptide induced PBMC proliferation.

PCP1 (PEG coated AuNPs conjugated with peptide): 3.6µg/mL PEG, PCP2: 8.4µg/mL PEG, PCP3: 16.8µg/mL PEG and PCP4: 25.2µg/mL PEG coated gold nanoparticles conjugated with peptide.

Gold nanoparticles coated with PEG are also tested but their values are near about same as of the blank. So the immunogenic potential of those samples was very less.

As concluded from the above graph PCP2 has good results so in second trial only 8.4µg/ml PEG coated gold nanoparticles conjugated with peptide, ConA (as a positive control), peptide (P_N5) were tested. In Figure 5.4.2. average of stimulation index of both experiments was taken.

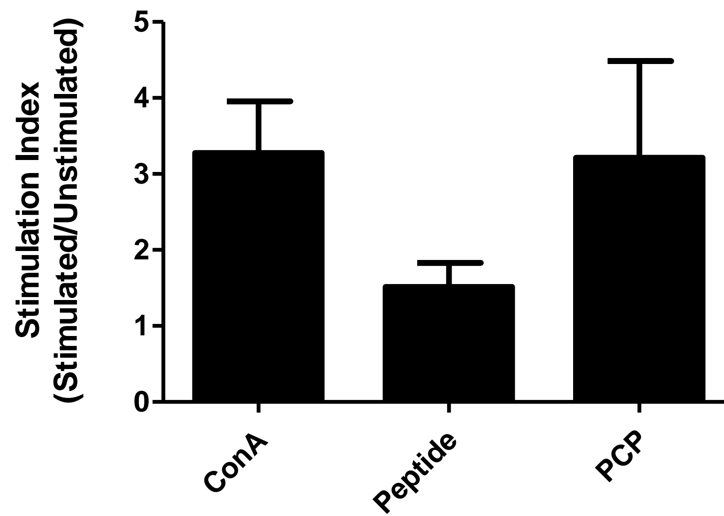


Figure 5.4.2. Neuraminidase peptide induced PBMC proliferation. Significant differences between unstimulated vs synthetic peptide stimulated cells are indicated by ($p<0.05$).

5.5. Conservancy analysis of N5 Peptide

The conservancy of the N5 peptide was checked in different subtypes of influenza virus. It was observed to be 98.4 and 95.7% in H1N1 and H5N1 respectively.

Table 5.5.1: Conservation of Neuraminidase peptide in H1N1 subtypes

	Peptide	Virus	Subtypes	Conservation %
P_N5	GSFVQHPELTGL	Influenza virus	H1N1	98.4%
			H5N1	95.7%

Chapter 6

Discussion

Influenza is a fast communicable infection of the respiratory tract and become pandemic if not properly cured. Various conventional influenza virus vaccines are available, but they are successful against limited strains of influenza infection so we need an innovative approach which is effective against different strains of influenza virus.

In this study we explored the effect of bio-nanocojugates on immune response. The peptide (GSFVQLPELTGL) has been predicted and evaluated in our previous study which has conducted in our lab [Lohia et. al., 2015].

The synthesis of gold nanoparticles was successfully done at room temperature. For it UV-visible spectrophotometer was used. A curve will form between 500-600 nm which indicates formation of gold nanoparticles. The size of nanoparticles was checked by Dynamic Light Scattering (DLS) method.

Poly ethylene glycol has been chosen among the most prominent polymeric nanoparticle used to manufacture drug delivery system and tissue engineering applications [Hirenkumar et. al., 2011]. In our study we use different concentrations of PEG (3.6, 8.4, 16.8, 25.2 µg/mL), for the coating of gold nanoparticles. The coating of PEG on gold nanoparticles and conjugation with peptide was done at room temperature. The conjugation of molecules was checked by Fourier transform infrared spectroscopy (FTIR).

MTT assay has been used to measure the enhancement in immune response [Jain and Baranwal, 2019]. By which we found that the proliferation of PBMC was high at 8.4µg/mL concentration of PEG coated gold nanoparticles conjugated with peptide. In a study different PEG concentration used for coating of gold nanoparticles which has been shown the fine results in drug delivery [Zhang et. al., 2012].

The conservancy test is required for checking antigenicity of peptide among all strains of pathogen [Lohia et. al., 2015]. The conservancy of the peptide sequence of Neuraminidase (N5) protein of H1N1 Influenza virus was checked using immunoinformatics tool. We found the H1N1 was 98.4% and H5N1 was 95.7% conserved.

Chapter 7

Conclusion

Peptide combined with nanoparticles has shown good potential in molecular interactions, vaccine and drug delivery system. Polymeric as well as metallic nanoparticles can be used as bio-conjugates to enhance the biological functions of peptides. A range of characterizations were done for the synthesis and conjugation of the nanoparticles with peptides. Neuraminidase peptide conjugated with PEG coated gold nanoparticles have shown enhanced proliferation as compared to peptide alone confirming the enhancement in immune response due to the nano-conjugates. The results were significantly good which shows this bio-nanocojugates are good candidate for stability and delivery of peptides. Hence, it is suggested that this PEG coated gold nanoparticles can be used as nano-adjuvants in vaccine formulation which further need to be validated in animal model.

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Conjugation of H1N1 Influenza peptides with PEG coated gold nanoparticles to enhance the immune response

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