Evaluation Of Safety And Therapeutic Profile Of Drug Loaded Influenza Virosome- *In Asthmatic Models.*

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Dedicated to My Parents

for being my passionate supporters, giving me strength by their trust and making me what I am today.

Amna Zahra

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In the name of Allah, the Most Gracious and the Most Merciful

Alhamdulillah, all praises to Allah, the Almighty, on Whom ultimately we depend for sustenance, guidance and strength. And may peace and blessings be upon our beloved Prophet Muhammad (*sallalahu 'alayhi wasallam*) for whom this world came into existence.

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Amna Zahra

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List Of Abbreviations

AIV	Avian Influenza Virus
AMA1	Domain I of Apical Membrane Antigen
C12E8	Octaethyleneglycol Mono(N-Dodecyl)Ether
CEA	Carcino-Embryonic Antigen
CIRIV	Chimeric Immunopotentiating Reconstituted Influenza Virosome
CPSF	Cleavage And Polyadenylation Specificity Factor
cRNA	Complementary RNA
CSP	Circumsporozoite Protein
CTLs	Cytotoxic T Lymphocyes
DCPC	1,2-Dicaproyl-Sn-Glycero-3-Phosphocholine
DEE	Diethyl Ether
DODAC	Di-Oleoyl Dimethyl-Ammonium Chloride
DTA	Diphtheria Toxin
EBV	Ebstein Barr Virus
EDTA	Ethylene-Diamine Tetraacetic Acid
elF	Eukaryotic Initiation Factor
Fab	Fragment Antigen Binding
FP	Fusion Protein
GP	Glycoprotein
HA Assay	Haemagglutination Assay
HCV	Hepatitis C Virus

HIV	Human Immunodeficiency Virus
IPA	Isopropyl Alcohol
IRIV	Immunostimulating Influenza Virosome
MAbs	Monoclonal Antibodies
mAbs	Monoclonal Antibodies
mRNA	messenger RNA
NA	Neuraminidase
NS	Non Structural
PABII	Poly-A Binding Protein II
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
PF	*Plasmodium falciparum*
PRI	Poultry Research Institute
PTHrp	Parathyroid Hormone Related Peptide
RBC	Red Blood Cell
RES	Reticuloendothelial System
RNP	Ribo-Nucleoproteins
Si RNA	Small Interfering RNA
SOP	Standard Operating Procedure
TAA	Tumor Associated Antigens
TSA	Tumor Specific Antigens
VSV	Vesicular Stomatitis Virus

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Abstract

Virosome technology presents a versatile carrier system with application extending in different fields of biomedical research. It has the potential to overcome major challenges of delivering therapeutically effective quantities of biological molecules to the targeted site. These reconstituted viral envelops not only guarantee targeted delivery but also ensure the protection and stability of the active biomolecules under different physiological conditions. On the bases of the success of virosome technology as delivering agent, the present study was designed to develop virosome containing drug and assess its toxicity as well as the efficacy. Influenza virus envelops were reconstituted along with the entrapment of drug (salbutamol sulphate) by the optimized protocol. Drug entrapment efficiency of these vesicles was calculated to be 84.95% and it was found that the drug levels were sustained in the samples for 96 hours. The toxicity of these vesicles was assessed in BALB/c mice. During toxicity assessment, no apparent signs of toxicity were seen physically. Moreover histology images of the lungs, liver, kidneys and spleen showed no difference when compared with the healthy tissue slides. The second part of the study was related to the development of asthma model with ovalbumin and alum in order to check the efficacy. Effects of drug encapsulating virosomes were analyzed and compared with the free drug and liposome using these diseased models. Different tests such as injection of β-blocker (Labetalol), differential leukocyte count, eosinophil count and challenge with the cigarette smoke proved the successful development of asthma model. The diseased models were then given the treatment of virosome, drug and liposome. By assessing the weight trends, pre-convulsion time and histology images it was found that the virosome showed comparable results with free drug and liposomes. The pre-convulsion time periods recorded for the virosome treated group showed the prolonged effect of the drug. The ease of the preparation of virosomes with encapsulated drug and the favorable results related to the toxicity and efficacy, provides a positive opportunity for increasing the half live and bioavailability of different drugs.

Chapter 1

INTRODUCTION

Virosomes are the reconstituted viral envelopes devoid of the genetic material (DNA or RNA). Virosomes are composed of membrane lipids with viral fusion proteins present on their surface. These viral spike glycoproteins, present on the virosomal surface, mediate the attachment and fusion activity of this virus like carrier system. The structure of virosomes is such that their external surface resembles the wild type virus with glycoproteins projecting from the membrane. In simple words these can be considered as empty viral particles. The main component of the virosomal wall is phospholipids, just like liposome. Virosomes can also be regarded as modified liposomes with embedded viral coat proteins. Their structure allows them to be used as carrier system and hence can be used to deliver antigens. Moreover, its inner empty compartment can protect the biomolecules from enzymatic degradation (Hunziker *et al*., 2001). Almeida et al., in 1975, was the first one to develop a virosome; immunopotentiating reconstituted Influenza virosome (IRIV) were synthesized by purifying influenza virus fusion proteins (haemagglutinin HA and neuraminidase NA) and inserting them into pre-formed liposomes (Almeida, 1975). Following the success of formation of IRIV, many enveloped viruses were reconstituted such as rabies (immunosomes), vaccinia virus, HIV, EBV, sendai and vesicular stomatitis virus (VSV) (Helenius *et al*., 1981; Marsh *et al*., 1983; Cooper *et al*., 1984; Perrin *et al*., 1985; Metsikko *et al*., 1986; Cornet *et al*., 1990; Bagai and Sarkar, 1993; Grimaldi *et al*., 1995). For adequate antigen presentation, reconstitution of particular virus should take place in a proper manner so that they acquire the characteristics of wild type virus (Ollivon *et al*, 2000).

Influenza virus is one of the most commonly used viruses for the production of virosomes. Influenza virus is an enveloped virus with single stranded, negative sense RNA genome and belongs to *Orthomyxoviridae* family. Its genome comprises of 8 segments and is liable to spontaneous mutation (Rambaut *et al*., 2008). Influenza virus causes a disease, which is commonly known as flu, in birds and mammals. It is broadly classified into three types, namely A, B and C based on the antigenic property of two viral fusion proteins (HA and NA) (Sato *et al*., 2010). Influenza type A and B are of major concern as type C is not related to significant human ailment. Influenza A virus is divided into further subtypes on the basis of nature of the envelope glycoproteins, haemagglutinin HA (H1-H16) and neuraminidase NA (N1-N9). Notable outbreaks of varying extent of influenza occur consistently. This epidemiologic pattern shows the fluctuating nature of antigenic properties of the virus. Specifically influenza A virus has a remarkable capability to undergo occasional antigenic changes in the envelope glycoproteins (Gamblin *et al*., 2010). The two main trans-membrane glycoproteins of influenza virus are haemagglutinin (HA) and neuraminidase (NA). HA facilitate virus attachment to the cell sialic acid receptors and virus fusion, while NA cleaves off the sialic acid and facilitates virus budding. The function of both these receptors is crucial for the virus replication and transmission (Zhu *et al*., 2012). Influenza virus HA recognizes the sialic acid receptor present on the host cell. Sialic acid is present on the cell surface, linked to the surface glycoproteins and gangliosides. It is recognized by many viruses, such as mumps, corona, rota and DNA tumor viruses, as a receptor for cell entry and is widely distributed in many organisms (Matrosovich *et al*., 2013). In humans, it is present on red blood cells, cell membrane of upper respiratory tract and excessively in the brain cells (Wan and Perez, 2006).

Among all the enveloped viruses used for the production of the virosome, Influenza virus is the most frequently used virus. Influenza virosome contains an outer shell of phospholipids shell with embedded influenza virus peplomers (Haemagglutinin and/or Neuraminidase). As mentioned above HA is majorly involved in the recognition of receptor and membrane fusion while virus budding is mediated by NA (Tumpey *et al*., 2007). The mean diameter of virosome is about 150nm (Huckriede *et al*., 2005). Virosomal surface display the envelope glycoproteins of a specific virus and thus can elicit humoral immune response. This property of virosome can be exploited for its use as vaccine agent. Moreover, virosome can also facilitate the safe and targeted transport of biomolecules as its surface glycoproteins have preserved receptor recognition and membrane fusion activity (Felnerova *et al*., 2004).

Several virosomal products have gained regulatory approvals and are now available in the market, while many related products are under pre-clinical and clinical trial phases. Two virosome based vaccines, that is Inflexal and Epaxal, are available in the market containing glycoproteins related to Influenza virus and Hepatitus A virus respectively (Mischler and Metcalfe, 2002; Bovier, 2008). Several others, such as Diphtheria vaccine, Tetanus toxoid vaccine, PEV 6 A and Peptido-mimetic vaccine AMA1 from PF are also undergoing clinical trials. The first three products contain antigens coupled to Influenza virosome and are directed against Diphtheria, Tetanus and breast cancer respectively (Felnerova *et al*., 2004; Wiltschke *et al*., 2008), whereas the Peptido-mimetic vaccine contains conserved epitopes of the domain I of apical membrane antigen (AMA-I) which were exploited against the malaria parasite. (Mueller *et al*., 2003). On the other hand, the products under pre-clinical trials are against breast and ovarian cancers, AIDS and rabies (Hunziker *et al*., 2001; Waelti *et al*., 2002; Bomsel, 2011).

According to pharmacological demands, an ideal drug molecule should be safe, cost effective and therapeutically efficient. One of the most common problems that is encountered during drug development is related to its stability under different physiological conditions. Many potential biomolecules are discarded because of this stability issue, as an adequate quantity of drug is required to reach the targeted cells in order to ensure the bioavailability at the site of action (Patel *et al*., 2010). The field of drug delivery technology has evolved significantly in the recent years due to the significant contributions made by many researchers. These studies have provided better understanding of the physiological barriers encountered by the bioactive molecules owing to their movement through circulatory system, cells and tissues. All these barriers play some of the part against the efficient drug delivery to the targeted cells. Due to these advancements there are now several novel drug delivery systems under clinical trials. Thus far, many potential drugs are often discontinued during the developmental procedure because of the lack of appropriate delivery systems and adverse effects due to the interaction with other non-targeted body cells and tissues (Chopra, 2006). Thus efficient engineered technologies are required to ensure the targeted delivery and controlled release of the therapeutic agents. Regardless of the research, the major problem in the efficient delivery of therapeutic molecule into the cytoplasm of the targeted cell is to overcome the permeability barrier of the plasma membrane (Phillips, 2001). For this reason many drug delivery systems have been formulated and are being investigated, including virosomes, liposomes, proliposomes, microspheres, prodrugs, cyclodextrins etc (Tiwari *et al*., 2012). All these delivery systems have their merits and demerits. (Saravanan *et al*., 2010)**.**

Liposomes are being used as a carrier system for targeted delivery of these biologically active molecules. They have gained acceptance as a successful carrier system in the field of

pharmaceutical technology. In spite of the considerable evolution in the liposomal technology, its commercial success is still restricted due to incompetent gene transfer and in-vivo expression (Felnerova *et al*., 2004). One of the major hurdles encountered by liposome is difficulty in fusing with the endosomal membrane due to which the encapsulated molecules are not efficiently delivered to the cytoplasm of the targeted cell. Moreover, liposomes are rapidly cleared from circulation as they are taken up by reticuloendothelial system (RES), mainly in the liver. Virosomes can easily overcome these issues as they adhere efficiently to the targeted cell due to the presence of functional envelope glycoproteins, which possess receptor binding and fusogenic properties, ensuring the cytoplasmic delivery of the molecules (Kaneda *et al*., 2000; Gowtham *et al*., 2012; Kalra *et al*., 2013).

A delivery system presented by virosome technology has the potential to transmit biological molecules, genetic material, protein antigens, etc. to the targeted site. It not only guarantees targeted delivery but also protect the active agents from endo-lysosomal degradation. As it ensures the delivery of genetic code -DNA or RNA - coding for specific immunogen, it can be efficiently used for vaccination purposes. Furthermore, the specificity of virosomes can be enhanced by coupling antibodies, specific to certain receptors, to its surface (Mastrobattista *et al*., 2001). This property of virosomes is exploited for delivering the drug molecules with narrow safety profiles (Kannan *et al.,* 2006).

Virosome is a versatile carrier system with its application extending in different fields of biomedical research. It is being used in the field of cancer therapies, delivery systems for drugs, genes and siRNAs, and also as vaccines or as adjuvants. Its demand is increasing day by day with its progress. In cancer research, engineered virosomes are being used based on the principles of cancer immunotherapy (Adamina *et al*., 2006). Moreover, virosomes have been

used to carry peptides corresponding to tumor specific antigens (TSA). Various tumor associated antigens (TAA) have been identified for instance HER2/nu, CEA, MAGE and WTI (Kaneda *et al*., 2013). Virosomes, as the delivery agents of genes, also have a potential to be used for the treatment of genetic diseases such as cystic fibrosis, hemophilia B (liver-derived factor IX), hereditary tyrosinemia type I and other genetic disorders of skin and muscle (Sarkar *et al*., 2002). Recently, influenza virosome has also been established as an efficient means of carrying siRNA to cells. SiRNA complexed with cationic lipids can easily be incorporated into the virosome (Huckriede *et al*., 2007). Virosomes have been investigated for the delivery of peptides and nucleic acid based vaccines for several diseases including malaria, melanoma, HCV, and Alzheimer's disease (Kaneda *et al*., 2000). Virosomes can also be used as adjuvants in human vaccines. Although their role is to enhance the immunogenicity of antigens, they are also used in order to get more specific and enhanced effects (Hagan and Derek, 2007).

Current study is divided into two parts; the first involves the constitution of virosome and second part is related to the development of an asthma model. Asthma is a chronic inflammatory disease that involves airways in the lungs. Characteristic features of asthma include inflammation, persistent airway hyper-responsiveness and reversible airway obstruction (Asher *et al*., 2006). It causes structural alteration in the airway for example airway wall fibrosis, goblet cell metaplasia/hyperplasia, smooth muscle thickening and increased vascularity (Alvarez *et al*., 1992). In other words, the asthma results in 'airway remodeling' and may result due to the repeated exposure of an allergen (zosky and Sly, 2007). The exact cellular and biochemical mechanisms underlying chronic inflammation and airway remodeling are poorly understood and there is a need to not only investigate these mechanisms but also to identify the pathways and novel targets for drug therapy. These investigations cannot be performed on humans owing to

ethical issues (Bice *et al*., 2000). Animal models provide an alternative for investigating inflammatory processes and alteration in the airway taking place during the disease.

Asthmatic mouse models have a fundamental place in asthma research. Owing to the complex and diverse nature of this disease, it is highly unlikely that a single mouse model can be regarded as fully representative of the disease. Usually each model is developed in order to mimic specific phenotype of asthma. Acute allergen challenge models have been widely used to study airway hyper-responsiveness and pulmonary inflammation but show some limitations when characteristics related to chronic asthma are considered. On the other hand, chronic allergen challenge models appear to mimic some characteristics of chronic asthma related to pathogenesis of the disease. However, both acute and chronic allergen challenge models have certain restrictions that need consideration when extrapolating results from animal model to human disease (Barlett *et al*., 2008). During the past research, mouse models have proved to be suitable for imitating clinical asthma up to certain extent and still different studies are continuing to improve the existing models in order to improve their usefulness. For instance, asthma exacerbations are becoming a focus of research as it is related to many asthma patients. Mouse models are being investigated for developing a model capable of reproducing asthma exacerbations. The researchers are working on both acute and chronic allergen model in order to develop improved asthma model which will more closely reflect human disease (Siegle *et al*., 2006; Ito *et al*., 2008). All these advancements will expand our awareness about the disease and will assist in identifying the novel therapeutic targets.

In order to develop asthmatic models, different allergens can be used, for example ovalbumin (OVA), house dust mite HDM, cockroach allergens (CRA), ragweed and fungi (Kurup *et al*., 1997; Barrett *et al*., 2003;Fishbein *et al*., 2016; Li *et al*., 2016). The most commonly used

allergen is OVA which is used in most of the studies to sensitize and challenge the host model owing to its inexpensiveness and availability in highly purified form. Purified form of OVA is free from protease or endotoxins which are found in other allergens. Moreover, the immunodominant epitopes of OVA are well-characterized and recombinant peptides have been generated. One of the drawbacks of OVA is that its repeated exposure may induce tolerance in the targeted model rather than developing a chronic allergic response. OVA is usually given in combination with an adjuvant for sensitization purpose. The sensitization process also includes booster doses of the OVA-adjuvant injection. The sensitization step is followed by the challenge step, which includes the series of inhaled or intranasal challenges in order to elicit an immune response.

Considerable concerns occur when assessing the results from the studies in OVA-challenge asthma models. These issues are related to the approaches and techniques employed for evaluation of response in models which cannot be corresponding to human assessment. Again at this point numerous alternatives are considered. Therefore, when studying the animal model, different parameters are taken into account such as inflammation, airway hyper-responsiveness, airway remodeling and histology. All these parameters are studied for understanding the disease progression and characteristics.

On the basis of the success of virosome technology as delivering agent, the present study was designed to assess the toxicity as well as the efficacy of drug containing virosome. It is based on the results obtained from the optimization and characterization of the drug encapsulating influenza virosome obtained from our previous research. This study aims to reconstitute influenza virus envelop along with the entrapment of drug, salbutamol sulphate (asthma drug), by the same optimized protocol so as to maintain the size as well as the characteristics. The

second part of this project is to test the toxicity of the developed virosome. Virosomes are composed of phospholipids and viral coat proteins. Phospholipids are considered safe for pharmaceutical use after testing, while the viral coat proteins and other constituents added to increase stability or enhance the properties of virosomes, may give them toxic potential. The study was limited to common *in vitro* screening tests and general outline of tests required for drug registration. Third part of this project was to test the efficacy of the drug containing virosome.

Present study was designed to achieve the following specific objectives:

- To constitute influenza virosome with encapsulating drug (salbutamol sulphate).
- To establish the drug entrapment efficiency and drug release profiles.
- To investigate toxic potential of virosome in BALB/c mice, *in-vivo*.
- To develop acute allergen challenge asthma model using ovalbumin and alum (adjuvant) for assessment of efficacy.

Chapter 2

LITERATURE REVIEW

2.1 VIROSOMES

Virosome are reconstituted lipid based vesicles comprising of natural coat proteins and functional fusion glycoproteins of normal virus. These are the carrier vehicles with unilamellar lipid membranes with intercalated viral surface proteins and hence resemble viral particles. These particles lacks replication property as well as pathogenicity with the retention of their immunogenic potential. The synthetic nature of the particles provides an inner cavity for incorporation of various therapeutic agents and can be used as a vehicle for delivering biomolecules (Kalra *et al*., 2013). The concept of virosome was first presented by Almeida, who prepared immunostimulating reconstituted influenza virosomes (IRIVs) by inserting influenza virus surface proteins i.e. haemagglutinin and neuraminidase; into preformed liposomes. The resultant particles resemble the original virus (Almeida, 1975). Ever since, virosomes of several different viruses, such as rabies (immunosomes), vaccinia virus, human immunodeficiency virus (HIV), Ebstein Barr virus (EBV), sendai and vesicular stomatitis virus (VSV), have been developed (Helenius *et al*., 1981; Marsh *et al*., 1983; Cooper *et al*., 1984; Perrin *et al*., 1985; Metsikko *et al*., 1986; Cornet *et al*., 1990; Bagai and Sarkar, 1993; Grimaldi *et al*., 1995).

Virosomal production is the emerging technology that possesses the characteristics of a widely applicable adjuvant as well as carrier system. Moreover, virosome based vaccines has shown high efficacy and high safety profile and can be regarded as safe for the children as well as the

immuno-compromised patients. It ensures targeted delivery of the antigens. A number of virosome based products are currently under investigation. Table 2.1 presents the various viruses that have been used for preparation of virosomes.

Table 2.1: Various viruses used for the preparation of virosomes.

Virus Name	**Surface Antigen Used**	**Reference**
Influenza Virus	HA (haemagglutinin), NA (neuraminidase)	(Almeida, 1975)
Rabies Virus	G protein	(Perrin *et al*., 1985)
Semliki Virus	E1, E2 glycoprotein rosette	(Helenius *et al*., 1981) (Marsh *et al*., 1983)
Human Immunodeficiency Virus	gp120, gp41	(Cornet *et al*., 1990)
Ebstein Barr Virus	Gp85	(Grimaldi *et al*., 1995)
Sendai Virus	F (fusion protein) HA-NA protein (haemagglutinin-neuraminidase)	(Bagai and Sarkar, 1993)
Vesicular Stomatitis Virus	G protein	(Metsikko *et al.*, 1986)
Respiratory Syncytial Virus	F (fusion protein) G (attachment protein)	(Kamphuis, 2012)
Sindbus virus	E1, E2 glycoproteins	(Scheule and Rounald, 1985)

2.2 INFLUENZAVIROSOME

Influenza virus is one of the most commonly used viruses for the production of virosomes. It belongs to *Orthomyxoviridae* family. It is an enveloped virus with single stranded, negative sense RNA genome. Influenza virosome contains virus hemagglutinin (HA) and neuraminidase (NA) inserted into the lipid membrane. Fundamentally, these vesicles are reconstituted empty influenza virus envelopes, devoid of all the genetic material of the natural virus. The biologically active surface glycoproteins are responsible for the unique properties of the virosome. The viral proteins are known to provide structural stability as well as contribute towards the immunological properties of virosome (Moser *et al*., 2013).

2.3 STRUCTURE AND COMPOSITION OF VIROSOME

Virosomes are unilamellar vesicles with a mean diameter of about 150nm (Noori *et al*, 2011) (Hunziker *et al*., 2002). The virosomal membrane is composed 70% of the naturally occurring phospholipids and 30% of phosphatidylcholine (Cusi, 2006). Lacking the genetic material, these particles are unable to replicate yet they retain their fusogenic activity. In comparison to liposome, these vesicles additionally contain functional viral envelope glycoproteins (HA and NA) inserted within phospholipid bilayer. The nature of the lipid bilayer determines the additional characteristics of virosome i.e. by varying the type or content of the membrane lipids, structure of the virosome can be optimized to attain the maximal incorporation of the molecules and show the best physiological effects. The structure of virosome provides variety of means for antigen deliver. The antigens can be incorporated into the virosome, adsorbed on the virosomal surface or integrated into the lipid membrane (Kalra *et al*., 2013). Moreover, the formulation and composition of virosome allows the incorporation or encapsulation of diverse nature. For

example, the lipid bilayer can easily integrate the hydrophobic drugs in it, whereas, hydrophilic drugs become a part of the central lacunae (Babar *et al*., 2013).

2.4 TARGETED DRUG DELIVERY

The current field of pharmaceutics demands the drug to be safe, reliable, efficient and cost effective while exhibiting targeted delivery and controlled release. The success of every drug is calculated on its stability, compliance, ease and most importantly its bioavailability i.e. reducing the amount of the drug needed for patient (Patel *et al*., 2010). Since long, drugs have been known to play a role in improving and extending life. However, the practice of drug usage has evolved during the last few decades. The efficiency of targeted drug delivery is hindered by a number of physiological barriers that it encounters during the transport in circulatory system and movement through cells and tissues. Researchers are continuously contributing towards the understanding of these physiological barriers and also to the development of novel drug delivery systems. Many such systems are presently in clinical practices. Nevertheless, many potential drugs are often discontinued during the developmental procedure because of the lack of favorable delivery system and intolerable side effects, occurring due to the interaction of other non-targeted body cells and tissues. There is a need to develop efficient engineered technologies which can be used for the targeted delivery and controlled release of the therapeutic agent.

There is a requirement of efficient delivery system that should be capable of targeting the drug to specific host cells and tissues. This drug delivery system should be used not only for the treatment of human diseases as well as the tool for studying the drug efficacy in the living cells. Regardless of the research and development in the viral or non-viral delivery systems, the major problem is the efficient delivery into the cytoplasm of the targeted cell is to overcome the

permeability barrier of the plasma membrane. For this reason many drug delivery systems have been formulated and are being investigated. These include virosomes, liposomes, proliposomes, microspheres, prodrugs, cyclodextrins etc (Tiwari *et al*., 2012). All these delivery systems have their merits and demerits (Sharma and Yasir, 2010)**.**

2.5 LIPOSOME VERSUS VIROSOME

Since long, liposomes have been used for the targeted delivery of bio-active molecules and have become a recognized carrier system in the field of pharmaceutical technology. They are being used in the treatment of various infectious diseases, cancers, autoimmune disorders as well as hormonal dysregulation. Despite the significant progress in the liposome technology, its commercial success is still restricted. The major hurdle to its successful application is inefficient gene transfer as well as expression *in vivo* (Felnerova *et al*., 2004). The liposomes fuses with endosomal membrane less efficiently and thus cannot significantly deliver the encapsulated molecules to the cytoplasm of the targeted cell. Moreover, liposomes are rapidly recognized and cleared by reticuloendothelial system (RES) from the circulation. In order to meet all of these challenges, virosomal technology presents a novel sophisticated delivery system. Virosomes, due to the presence of envelop glycoproteins, bind with the targeted cell more efficiently and ensure the delivery of the molecules into the cytoplasm, because envelop glycoproteins are functional and retain their receptor binding and fusogenic properties (Kaneda, 2000; Gowtham *et al*., 2012; Kalra *et al*., 2013). In recent studies, virosomes have been used for the delivery of drugs, genetic material and antigens. It is suggested that controlled release of various agents can be significantly improved by using virosome carrier (Langer, 1998).

2.6 ADVANTAGES OF VIROSOMES

Virosome technology represents the most practical carrier system, which has a range of potential applications for the prevention as well as the treatment of diseases such as cancer, infectious diseases and certain neurodegenerative disorders. Because of their non-toxic nature and high safety profile with no risk of disease transmission, virosomes have been approved by FDA. Virosomes are biodegradable and biocompatible. Virosomes protect the bioactive molecules from endosomal degradation as these vesicles undergo fusion activity in the endosomal pathway (Helenius and Fries, 1977). Additionally, virosomes facilitate the encapsulation of variety of pharmaceutically active compounds such as antigens, antibiotics, cytostatics, nucleic acids and other macromolecules. Moreover, surface of virosomes can also be modified according to the nature of the delivering compound. The other beneficial properties such as extended uptake, control release and less immunogenicity or anaphylaxis enables these delivering agents to be used as an efficient carrier system for the delivery of drugs and other macromolecules (Gowtham *et al*., 2012). The encapsulation of the bio-active molecules not only protects them from the degradation and ensures their delivery into the targeted cells but also protects the patient from the direct side effects of the drug (Kalra *et al*., 2013). In case of vaccination, virosomes depict the potential of both the specific delivery of antigens and the amplification of the immune response. Research has shown that virosomes are capable of stimulating both the arms of immune system i.e. humoral and cellular response. Besides, this technology also provides the opportunity of patient specific modular vaccine regimen (Miller, 2003; Gabbula and Chaitanya, 2013).

2.7 VIROSOME PREPARATION

The basic protocol of the virosome formation is same regardless of any type of virus. The procedure begins with the detergent solubilization of the viral membrane followed by the sedimentation of internal viral proteins and genetic material by ultracentrifugation. The next step includes the detergent removal which results in the reconstitution of phospholipids, membrane proteins and biological membrane (Daemen *et al*., 2005).

Antigens of a selected virus can either be incorporated onto the virosomal surface or into the inner empty compartment, as per the requirement and nature of the selected antigen. The selected antigen can be of any nature for example in case of vaccination, parasite, viral receptors, bacterium, carcinogenic cells or whole cell can be used as an antigen. While in case of delivery, cell components such as DNA, RNA, plasmids are used as antigens. The nature of the chosen antigen will determine its attachment to the virosome i.e. either it can be packaged inside IRIVs, integrated or anchored into the lipid bilayer, crosslinked onto HA or adsorbed to the membrane (Patel *et al*., 2010).

Solubilization is the first step in the reconstitution of virosome, which is carried out using detergent. Different detergents used for solubilization process include octaglucoside, triton X 100, nonidert p-40, C12E8 (octaethyleneglycol mono(n-dodecyl)ether), DCPC (short chain lecithin) (de Jonge et al., 2006; Sarachai *et al*., 2013). After solubilization, the next step is the sedimentation of the internal viral proteins and genetic material. The next step includes the removal of the detergent from the supernatant by dialysis or by using hydrophobic resins (SM-2 bio-beads). Then, ultracentrifugation method is used to remove viral matrix proteins and nucleocapsid. Along with the recovery of viral proteins, 82% of the phospholipid is also recovered. Now the antigen already coupled to lipid anchor is mixed with polymer or surfactant

solution and this solution is processed with virosome carrier so that antigen bound virosome is obtained (Stegmann *et al*., 1987; Bron *et al*., 1994; Kalra *et al*., 2013). Figure 2.2 exhibits the basic process of virosome preparation.

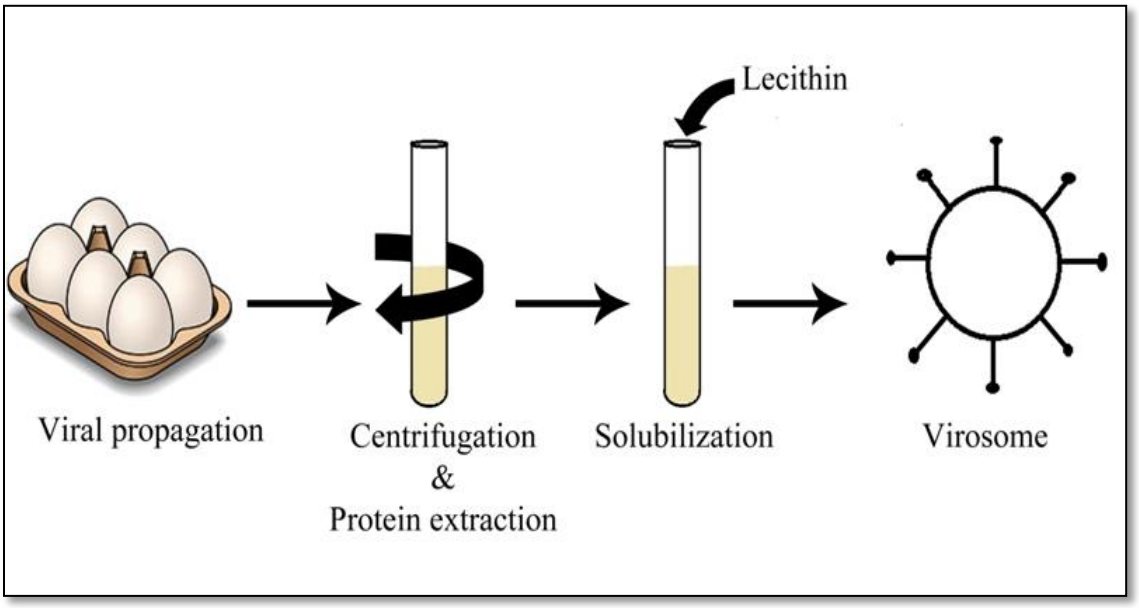

Figure 2.1: Process employed for the development of Virosomes

2.8 CHARACTERIZATION OF VIROSOME

The characterization of the virosomes focuses at the determination of the particle size, protein and phospholipid content and their biological properties.

2.8.1 Protein detection

For the determination of the protein content of the virosomal formulation different techniques are used which include sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE), Lowry assay or western blotting. These techniques signify the amount of protein antigen (HA, NA) and also confirm the quantitative removal of other non-membranous proteins (NP, M1). It is

generally accepted that virosome should show relatively uniform protein to lipid ratio (Peterson, 1977; Sarachai *et al*., 2013; Abdoli *et al*., 2013).

2.8.2 Size and structure

Transmission electron microscopy (TEM) can be used for analyzing the structural characteristics, size as well as purity of the virosome. The traditional methods are not successful for monitoring very small particles such as virus. Hence, negative stain EM can be used in order to analyze the size and ultra-structure of the virosome. The staining should be of neutral pH, so as to avoid the conformational changes of HA which are induced by acidic pH (de Jonge *et al*., 2006).

2.8.3 Sucrose density gradient assay

For detection of phospholipids, protein and DNA, virosomal formulation can be analyzed on a linear sucrose density gradient. The gradient are first centrifuged to equilibrium and then the fractions are analyzed for protein, phospholipid and DNA content (Jorgen *et al*., 2007).

2.8.4 Fusion activity

Different studies show that the membrane fusion activity exhibited by the virosome is similar to the fusion activity displayed by the native influenza virus. Fusion activity can be determined either directly or indirectly. In the direct method, the fusion of the virosome with the biological or artificial target membrane can be examined in vitro by excimer assay using pyrene-labeled lipids. The decrease of surface density of the pyrene-phosphatidylcholine-label on fusion with the unlabeled membrane results in the reduction of excimer fluorescence. In the other method, the fusion activity is indirectly analyzed by calculating the hemolytic activity, which corresponds

closely to fusion activity (pH dependence same as that of the fusion process) (Scheule, 1986; Fleddermann *et al*., 2016).

2.8.5 Haemagglutination assay

Haemagglutination assay can also be used for the biological characterization of the virosome. For example in case of influenza virosome the fusion activity can be visualized by HA test using chicken blood. Fusion activity of the virosome is confirmed by the absence of the button formed at the bottom of the well. Whereas the formation of the button due to the action of gravity represents negative results (Sarachai *et al*., 2013).

2.8.6 Infectivity and Toxicity assay of virosome

Cell lines can be used in order to evaluate the toxicity and infectivity of the constructed virosome. Different cell lines such as Vero (African Green Monkey kidney cells), MDCK (Madin-Darby Canine kidney cells), BHK (Baby Hamster kidney cells), etc. can be transfected with the virosome and then the cells are monitored for cytopathic effect for around 72 hrs (Jorgen *et al*., 2007; Abdoli *et al*., 2013).

2.9 APPLICATIONS OF VIROSOMES

Virosome technology provides an efficient and flexible carrier system that can be used for delivering DNA, RNA or antigens. In case of vaccines, DNA or RNA encoding for specific immunogen can also be delivered. It ensures targeted delivery of the antigen. In some cases the virosome can also be coupled to an antibody to increase the tissue specificity. These coupled antibodies recognize the specific receptors and deliver the antigen to the targeted cells. This property can, especially, be utilized for carrying the drug molecules with narrow safety profiles.

In addition to the targeted delivery, virosomes also protect the active agents from the endo-lysosomal degradation (Babar *et al*., 2013). A number of virosome-based products are under investigation for their therapeutic and prophylactic benefits. Table 2.2 summarizes these products along with the purpose they are used.

Table 2.2: Virosome products available in the market and under development

Virosome product	Antigen	Application	Reference
Products in the market			
Epaxal	Inactivated hepatitis A virus coupled to a virosome	Vaccination	(Bovier , 2008)
Inflexal	70% NA and HA 30% phospholipids	Virosome based trivalent influenza vaccine	(Mischler and Metcalfe, 2002)
Products under clinical trials			
Diphtheria vaccine	Subunit A of Diphtheria toxin A	Influenza virosome based vaccine	(Bron *et al*., 1994)
Tetanus toxoid vaccine	Tetanus toxin	Influenza virosome based vaccine	(Cusi, 2006)
PEV 6 A	Her2	IRIV coupled vaccine against breast cancer	(Wiltschke *et al*., 2008)
Peptido-mimetic vaccine AMA1 from PF	Loop I of domain III of AMA-1	Vaccination against malaria	(Mueller *et al*., 2003)
Products under pre-clinical trials			
Doxorubicin	Her2/Neu	Breast cancer, Ovarian Cancer	(Waelti *et al*., 2002)
HIV1 Simian Virosome	gp41	AIDS	(Bomsel, 2011)
Rabies immunosomes	Surface glycoprotein	Rabies	(Perrin et al., 1985)

2.9.1 Cancer treatment:

Currently, general cancer therapies include surgery, chemotherapy and radiation therapy, but all of these have limitations. Many groups are investigating ways to improve the conventional method for more effective cancer treatment with fewer side effects. Cancer immunotherapy is progressively being accepted as a choice for treating cancer that is in an advanced stage. The discovery of tumor-associated antigens in 1991 has accelerated the evolvement of antigen-specific immunotherapeutic techniques for a wide range of cancers. This therapy too has some limitations, which creates the need for further enhancements in the arena of immunotherapy. As versatile antigen carriers, virosomes can be engineered to carry out a plethora of tasks in cancer immunotherapy (Adamina et al., 2006). Influenza virosome was used for delivering the TAA for parathyroid hormone related peptide (PTHrp) in mice and was successful in eliciting immune response. Modified influenza virosome was also targeted to ovarian cell carcinoma (Correale *et al*., 2001; Mastrobattista *et al*., 2001). The other recombinant proteins include HER2/neu (overexpressed in breast cancer) combined with anti Fab–doxovirosome integrated the anti-proliferative characteristics of the monoclonal antibodies and cytotoxic response of doxorubicin *in vivo.* The virosome developed against metastatic breast cancer is in phase 1 clinical trial. A virosome-based vaccine designed using Her-2/neu multipeptide generates Her-2/neu specific immune effect in patients suffering from metastatic breast cancer (Waelti *et al*., 2002). The virosome has been used to carry peptides corresponding to TSA. Various TAA have been identified e.g. HER2/nu, CEA, MAGE and WTI (Kaneda *et al*., 2013).

Immunotherapy of virosome based vaccines have proved to be successful in treatment of cervical cancer. These virosomal based vaccine are being actively studied recently. Walczak and his coworkers demonstrated the effectiveness of combination therapy including virosome containing

HPV 16 E7 protein and recombinant Semiliki Forest Virus (rSFV) encoding fusion protein of E6 and E7 of Human Papillomavirus. This combination resulted in high antigen specific CTL response (Walczak *et al*., 2011). In another study the same combination was used with many T cell epitopes and endoplasmic reticulum signals. This study demonstrated the enhanced protein stability and strikingly increased frequencies of HPV- specific T cells (Bungener *et al*., 2006). Moreover, recent studies have shown the effectiveness of Hemagglutinating virus of Japan (HVJ). According to the recent studies HVJ envelope has certain antitumor properties i.e. it may be involved in induction of antitumor immune response and in the induction of apoptosis in tumor cells. It was found that these vectors activate interferon-related pathways which lead to induction of the apoptosis in the tumor cells. These vectors are proven successful in killing of prostate cancer cells *in vitro* as well as *in vivo* (Miyamoto *et al*., 2009; Tanaka *et al*., 2010).

2.9.2 Gene delivery

The membrane fusion protein, haemagglutinin, recognizes the fusion activity of virosome at low pH. In this case the low pH-dependent fusion reaction between virosomal and endosomal membranes is exploited to achieve efficient gene delivery to the target cells. Gene therapy requires an efficient and safe delivery system. The available viral and non-viral vectors are efficient, but safety issues and targeted cell delivery are still major concerns. The techniques present for the delivery of gene, such as naked plasmid or gene gun are also not satisfactory. There exists a clear need of new delivery system for DNA that can be administered at low doses (Daemen *et al*., 2005). Virosome delivery systems also have a potential to be used for the treatment of genetic diseases such as cystic fibrosis, hemophilia B (liver-derived factor IX), hereditary tyrosinemia type I, and other genetic disorders of skin and muscle (Sarkar *et al*., 2002).

Influenza virosome reassembled in the presence of dioleoyldimethylammonium chloride (DODAC) were used to accomplish the effective gene delivery to cultured cells. HA reconstituted in the presence of DODAC resulted in the plasmid binding to the virosome surface. Virosomes containing DODAC and plasmid DNA encoding for B-galactosidase linked to their surface showed successful transfection of BHK-21 cells. This study shows the potential of DODAC- containing virosome to be used as a unique approach in gene transfer along with the high level expression of the transgene (Schoen *et al*., 1999). Similarly, the N-[l,2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethylsulfate (DOTAP) containing influenza virosomes were exogenously loaded with plasmid DNA which codes for HA or fusion protein of mumps virus. This complex was used to induce strong neutralizing antibody and cytotoxic T cell responses (Cusi *et al*., 2000).

2.9.3 DNA/ RNA Delivery

Small interfering RNAs (siRNA) mediated gene silencing is one of the most powerful techniques to study gene function *in vitro*. It has considerable therapeutic potential though, its success depends on the availability of efficient carrier system for introduction into the targeted cells. Recently, influenza virosome was studied for the siRNA cellular delivery. SiRNA complexed with cationic lipids can easily be incorporated into the virosome. It demonstrates that virosomes can be used as the carrier system for cellular delivery of siRNA both *in vitro* and *in vivo* (Huckriede *et al*., 2007; Lund *et al*., 2010).

2.9.4 Antigen Delivery

Virosomes have the potential to be used as a system for the delivery of antigens for vaccination purpose. It has been investigated for the delivery of peptides and nucleic acid based vaccines for

several diseases including malaria, melanoma, HCV, Alzheimer's disease (Kaneda *et al*., 2000). Fusion activity of the virosome is of prime importance when cytosol delivery of antigen is required. Virosomes have proved to be an appropriate entity for the efficient elicitation of the antibody mediated immune responses against a specific antigen (epitope), as they provide additional immunogenicity and also protect the incorporated peptides and adjuvants. Moreover the outer surface of virosomes resembles an envelope of the virus they are derived from, so they elicit antibody responses against the virus they are derived from (Huckriede *et al*., 2003). Virosomes can also be used to include the dissimilar antigens onto the membrane for eliciting immune response. For example Epaxal Hepatitis A Virion (HAV) which is a virosomal vaccine currently present in the market, is influenza derived virosome. It has shown to elicit a strong stimulation of HAV-specific antibody response (Nerome *et al*., 1990). In another study, the fusion activity of influenza virosome was exploited for the delivery of subunit A of the Diphtheria toxin (DTA), which was encapsulated in influenza virosome. The complete inhibition of cellular protein synthesis showed the successful delivery of DTA into the cell cytoplasm (Bron *et al*., 1994).

Immunization experiment in mice were used to carry out the first studies investigating the potential of virosome to activate antigen-specific CTLs. Priming of cytotoxic T lymphocyes (CTLs) activity demands presentation of antigen through the MHC-I pathway. In this study influenza virosome was used as a carrier for synthetic peptides analogous to major murine CTL epitope from influenza virus nucleoprotein (NP). Virosome containing encapsulated NP-peptide proved to be efficient in sensitizing the target cells for the detection of influenza-specific CTLs induced by priming of mice with infectious virus. Intraperitoneal, intramuscular, and subcutaneous immunizations efficiently induced CTLs. The outcome of this study depicted that

fusion active virosomes have the potential as efficient delivery system for the induction of MHC class I mediated CTLs using non-replicating viral antigens (Arkema *et al*., 2000; Sharma and Yasir, 2010).

In another study, chimeric immunopotentiating reconstituted influenza virosome (CIRIVs) was used for sensitization of T cells during HCV chronic state infection. CIRIVs embody high peptide encapsulation power of liposomes in addition to the fusion activity of virosomes. This chimera was able to incorporate 30-fold more of the quantity of peptide as opposed to the current preparation method. The immunogenicity of CIRIVs was studied in transgenic mice. These mice were immunized with chimeric virosome containing HCV core123 peptide, which induced specific cytotoxic T cell response. It was emphasized that vaccine concoctions, including combinations of different HCV-derived CTL epitopes could serve the purpose of inducing not only a strong but also highly multi-specific CTL response. Hence, they have the potential to act as therapeutic as well as prophylactic T cell vaccines in humans (Amacker *et al*., 2005). Immunopotentiating reconstituted influenza virosome (IRIV) is used as Inflexal V, which is a licensed flu vaccine in Europe. It is a virosome based trivalent vaccine which has proved to be highly immunogenic and well tolerated in all age groups i.e. children, young adults as well as in elderly people (Mischler and Metcalfe, 2002; Hagan, 2007).

2.9.5 Virosomes as adjuvants

Virosomes can also be used as adjuvants in human vaccines. In order to accelerate the immune response against an antigen, inactivated vaccines usually contain an adjuvant. Although their role is to enhance the immunogenicity of antigens, they are also included in order to get more specific and enhanced effects (Hagan, 2007). The admirable properties of virosome as an adjuvant for

human vaccination have been verified and registered by most European, 16 Asian and American countries in commercial hepatitis A and influenza vaccines (Cusi, 2006). Virosomes are also being used for the research of HIV vaccine. Recently, a study was successfully conducted on immunization of non-human primates using HIV-1 gp41 subunit virosome which resulted in the production of protective mucosal antibodies (Bomsel, 2011). Moreover, a range of ligands such as peptides, cytokines as well as monoclonal antibodies (MAbs) can also be assimilated into the virosome and exhibited on the virosomal surface. Tumor-specific monoclonal antibody fragments (Fab) can be coupled to virosomes and this amalgamation is then aimed toward the specific tumor cells.

2.9.6 Virosome in Malarial Therapy

Malaria, one of the most devastating diseases of the world, is caused by *Plasmodium falciparum* (PF), which is responsible for significant mortalities all around the world especially in Africa. There is no effective vaccine available against this multi-resistant parasite. Recent studies have shown that it may be possible to exploit human monoclonal antibodies (mAbs) as a prophylactic treatment in order to prevent infections, most importantly invasion of the hepatocytes. These mAbs can be directed against the sporozoites, the parasitic stage transmitted by the mosquito bite. Virosomes, being flexible can be allied to complete protein sequences and also synthetic peptides. Moreover virosomes are known to elicit CTL response by antigen presentation through MHC-I pathway. All these properties illustrate that virosome can be used for the production of malaria vaccine. PF passes through several developmental stages during its life cycle and an effective vaccine should be able to target different antigens of these stages. Recent studies have shown the enormous possible implications of influenza virosome as antigen delivery technology

which provides the platform for the advancement of multivalent malaria subunit vaccine (Daubenberger *et al*., 2008).

In 1999, a research was conducted in which IRIVs were used to deliver SP66 synthetic peptide malaria vaccine. The SP66 synthetic peptide with terminal cysteine residues was attached to phosphatidylethanolamine through covalent bonds. This conjugate was then linked to the virosome via hetero-bifunctional cross linker, g-maleimodobutyric acid N-hydroxysuccinimide. This virosome assembly was then inoculated twice in BALB/c mice. It was predicted that the pre-immunity with influenza virosome will accelerate the humoral immune response in humans. ELISA was used to determine the antibody titer against the peptide (SP66 IgG). Results were contradictory, showing no effect of priming on the antibody titer of the immunized mice. Moreover the mice receiving virosome peptides demonstrated a more steady response with low antigen doses. This illustrated that virosome adsorption on the host cells is mediated by viral HA and anti HA antibodies may play a significant role in Fc receptor mediated virosome uptake and MHC class II presentation of antigens associated with virosome. The results signify the great potential of IRIVs for the design of molecularly defined combined vaccines directed against multiple antigens during the developmental stages of one parasite, as well as against several pathogens (Frank *et al*., 1999).

In another study, the conserved epitopes of the domain I of apical membrane antigen (AMA-I) were exploited for the vaccine development against the PF. AMA-1 is one of the most commonly used target for a malaria subunit vaccine. The virosome formulation of a peptide imitating the semi-conserved loop I of domain III, was successful in eliciting growth inhibitory antibodies against the parasite. A synthetic peptide through its N-terminus was conjugated to a derivative of phophatidylethanolamine and this conjugate was then incorporated into IRIV as a human

compatible delivery system. Both the linear and cyclic versions of the peptide antigen were successful in eliciting the antibodies against AMA-1. The antibody titer was confirmed by western blotting and immunofluorescence assays, using parasite lysates and blood stage parasites respectively. Results showed that all peptidomimetic mAbs generated were cross-reactive with AMA-1 expressed by the PF. The presentation of the peptide antigen in its native-like state on the virosome surface is the most important aspect of synthetic vaccine formulation. This virosome-peptide system could be exploited for the production of synthetic peptide multi-antigen malaria vaccine (Mueller *et al*., 2003).

Recently, a double blind study was also conducted to estimate the safety and immunogenicity of virosome formulation of the two malaria peptido-mimetics derived from PF, NPNA repeat region of the circumsporozoite protein (CSP) and loop I of domain III of merozoite AMA-1. The experiment was conducted with malaria semi-immune adults and children for 90 days. Vaccine formulation successfully elicited the immune response with no severe or serious side effects. Pain in the injection site was the only local adverse effect reported (Cech *et al*., 2011).

Another research study also demonstrated the induction of humoral immune response in the experimental models as a result of immunization with virosme-peptide formulations. Again, two virosomal formulations using peptide derivatives from CSP and AMA1 of PF were included in the experimentation. In phase 1 clinical trials, this formulation induced high titers of peptide-specific antibodies. A thorough immunological as well as functional analysis of the specific antibodies was conducted. Virosome-CSP formulation exacted a long term parasite-inhibitory antibody reaction in humans. Composite of this formulation with the second virosome-AMA-1 formulation did not show any interference between the immunogenicity of either peptides (Okitsu *et al*., 2007).

All these examples demonstrate the potential of influenza virosome as an adaptable, human-compatible antigen and drug delivery platform for the progression of multivalent subunit vaccine.

2.10 DEVELOPMENT OF AN ASTHMA MODEL

Asthma is known as the chronic inflammatory disease involving the airways in the lungs. It is characterized by the inflammation, persistent airway hyper-responsiveness, reversible obstruction of the airways (Asher *et al*., 2006). Asthma also includes the structural alteration in the airway such as airway wall fibrosis, goblet cell metaplasia/hyperplasia, smooth muscle thickening and increased vascularity (Alvarez *et al*., 1992). In other words the asthma results in 'airway remodeling' and may result due to the repeated exposure of a certain antigen (allergen). This repeated exposure is responsible for all the characteristic symptoms of asthma (zosky and Sly, 2007). It is assumed that chronic inflammation together with the structural changes contributes to the symptoms of asthma. The exact cellular and biochemical mechanisms underlying chronic inflammation and airway remodeling are poorly understood and there is a need to not only investigate these mechanisms but also to identify the pathways and novel targets for drug therapy. These investigations cannot be performed on humans owing to the ethical issues (Bice *et al*., 2000). Animal models provide an alternative for investigating inflammatory processes and alteration in the airway taking place during the disease. Due to the complex multifactorial nature of asthma, single animal model of asthma capable of replicating all of the morphological and functional features of asthma, is highly unlikely (Persson *et al*., 1997). Nevertheless, different animals can be used to model specific feature of the disease. The different animals currently being used as asthmatic models include guinea pigs, rats, mice, ferrets, dogs, sheeps, monkeys and horses (Fairbairn *et al*., 1993; Colasurdo *et al*,. 1998; Bautsch *et al*., 2000).

Mouse is most commonly used animal for the development of *in vivo* asthma models because it is a not a endangered species and hence presents wide variety of genetically altered inbred strains at relatively low cost (Kips *et al*.,2003).

Nowadays, asthmatic animal models have been widely used in order to understand the mechanism of disease, function of genes involved and cellular pathways as well as for the prediction of the safety profile of new drugs and chemicals. These animal models have contributed a lot in the advances in understanding the pathophysiology of the asthma (Karol, 1994). The animal based studies have shown the association of asthma with cellular inflammatory response and gave us the details of contributors which play role in the characteristic symptoms. The studies done on the animal models have shown the involvement of not only the eosinophils but also describe the T helper cell functional heterogeneity and the specific cytokines profile (Kips *et al*., 2003).

2.10.1 Why asthmatic mouse model?

Asthmatic mouse models offer extensive advantages when compared to the other animals. One of the major advantage of using mouse as asthma model is that IgE is produced as primary allergic antibody and thus making it suitable for examining the function of humoral immune factors involved in the development of asthma. Additionally numerous immunological reagents, namely growth factors, cell surface markers and antibodies against cytokines are easily available for the mouse models which give us the opportunity to investigate the underlying mechanism of allergic reactions. Furthermore, well-characterized inbred strains of mice are abundantly available in the market. The new promising technology, such as gene manipulation in animals, is well understood in mice. For that reason, key progress in the understanding of the disease

"asthma as Th2-dominent disease" appeared from the studies in mice. The other common advantages of using mouse as a model includes its small gestation period which allow us to study the effect of disease in generations in relatively short time. Moreover, mice are small and inexpensive. On the other hand significant physiological differences can be seen between mice and humans. These differences sometimes becomes a cause of failure in interpretation of results from mouse to clinical settings. Mice models do not develop smooth muscle hyperplasia and spontaneous airway hyper-responsiveness (AHR) easily. Moreover they do not develop airway constriction response to histamines at all. One of the major limitations in mouse model as well as the other animal models is the incapability of developing a chronic asthma model (Clarke *et al*., 2014). Similarly, the other animals mentioned above, that can be used as asthma models, have merits and demerits (Michoud *et al*., 1978; Lowell, 1990; Ricciardolo *et al*., 2008).

Here we are keeping the focus of our study to mouse models only. For designing a study involving animal model certain things should be kept in mind which include the strain of mice, the allergen of choice, experimental protocol and outcome measures. Under the light of these four parameters an appropriate mouse strain should be selected. The parameters of allergic airway inflammation and airway hyper-responsiveness varies greatly among different species of mice. According to the literature the type of the mice C3H/HeJ and DBA/2 mice fail to develop allergen-induced AHR (Ewart *et al*., 2000). While, AKR/J and A/J mice show high level of allergen-induced AHR as well as reactivity to methacholine (Mclntire *et al*., 2001). Literature shows that the most widely used mice strains include BALB/c and C57BL/6 because of their well characterized immunological responses. C57BL/6 mice exhibit Th-1 dominated immune responses and hence show limitations in development of allergic airway response (Morokata *et al*., 1999). In contrast, BALB/c mice exhibit Th2 dominant immune response. These also causes

the induction of the parameters involved in allergic responses such as eosinophilic airway inflammation, AHR and allergen specific IgE around the airway. BALB/c mice are also sensitive to methacholine (Takeda *et al*., 1997; Fuchs and Braun, 2008).

2.10.2 Allergens for development of asthma models:

For developing asthma model many allergens can be used such as ovalbumin (OVA), house dust mite HDM, cockroach allergens (CRA), ragweed and fungi (Kurup *et al*., 1997; Barrett *et al*., 2003; Fishbein *et al*., 2016; Li *et al*., 2016). The allergen OVA is used in most of the studies to sensitize and challenge the host model owing to its inexpensiveness and availability in highly purified form. Moreover, the immunodominant epitopes of OVA are well-characterized and recombinant peptides have been generated. Purified form of OVA is free from protease or endotoxins which are found in other allergens. One of the drawbacks of OVA is that its repeated exposure may induce the tolerance in the targeted model rather than developing a chronic allergic response. OVA is usually given in combination with an adjuvant for sensitization purpose. The sensitization process also includes the booster doses of the OVA-adjuvant injection. The sensitization step is followed by the challenge step which includes the series of inhaled or intranasal challenges in order to elicit an immune response.

One research reports that in order to induce Th2 response to inhaled allergen, low level of lipo-polysaccharides (LPS) signaling is necessary. While the inhalation of high level of LPS results in TH1 response. According to this research the levels of LPS present in the OVA determines the nature of the immune response and explain the relation between the endotoxin exposure and asthma prevalence (Eisenbarth *et al*., 2002). While another research investigated the effect of repeated exposure of an allergen on airway inflammation and humoral responses in an already

sensitized mice. This study demonstrated that the repeated exposure of antigen alone does not result in the persistent infection but resulted in the tolerance i.e. reduced level of eosinophils (Swirski *et al*., 2002). Recently many different studies are focusing on the effect of purified proteins derivative of other human allergens such as CRA, HDM, ragweed, etc. on mice and other speies (Warner *et al*., 1930; Lambert *et al*., 1999; Blease *et al*., 2001; Chapoval *et al*., 2002). These allergens are notorious for inducing asthma attacks in humans and according to some studies these allergens also have illustrated their ability to cause chronic airway inflammation in mice. Different studies demonstrated that following the same protocol with different allergens elicit distinct responses in particular species (Seitzer *et al*., 2005).

Generally, during the development of animal model, the animal is systematically sensitized to allergen via intra-peritoneal injection of allergen along with an adjuvant and is challenged to allergen via the airways. According to the literature there are two types of asthmatic mouse models being developed, namely acute allergen challenge model and chronic allergen challenge model.

2.10.3 Acute allergen challenge model:

Studies have shown that asthma is not instinctively developed in mice; so in order to carry out the research of examining the disease, a mock asthmatic-like reaction is induced in the airways. Acute allergen challenge models are widely used to investigate the fundamental processes involved in immunologic and inflammatory responses in asthma. These models are also being widely accepted for the recognition and exploration of novel targets for controlling the allergic responses. As mentioned previously that the nature of the inflammatory model is influenced by the four factors. BALB/c mice most frequently used strain for developing allergen challenge

model as they elicit good Th-2-biased immune response (Boyce and Austen, 2005). On the other hand the strains C57BL/6 anA/J have also been used successfully in allergen challenge studies (Kumar *et al*., 2008). When talking about the allergens OVA derived from chicken eggs is commonly used allergen which has proven to be very effective in eliciting allergic pulmonary inflammation. According to a study, OVA is rarely involved in human asthma and many groups are working on other alternatives such as HDM and cockroach extracts, that may have greater clinical relevance (Sarpong *et al*., 2003; Johnson *et al*., 2003).

However many different sensitization and challenge protocols for developing an asthma model have been established, yet they all follow the same general path i.e. the sensitization step and challenge step. The protocol of acute sensitization typically involve multiple systemic administration of allergen in the presence of an adjuvant. Adjuvant play an important role in eliciting the immune response for example aluminium hydroxide ($AlOH_3$) used as an adjuvant, promote the development of Th2-response. On the other hand adjuvant free protocols require larger number of exposures in order to get the required level of sensitization (Blyth *et al*., 1996).

A study showed the effectivity of sensitization via airways using OVA and HDM. OVA models success was limited due to the diffident pulmonary inflammation and mild AHR. There may be a chance that antigens given via inhalation without prior systemic sensitization give rise to tolerance. While in case of HDM, as this protein has intrinsic enzymatic action, it was successful in inducing the immune response. Sensitization period usually cover a period of 14 to 21 days which involve the period of antigen exposure at regular intervals, in order to create immune memory. Antigen challenge can be given in many different ways such as it can be inhaled as aerosols, or given via intra-peritoneal injections, or directly administered by intra-tracheal (i.t) or intra-nasal (i.n) instillation in an aqueous form (Foster *et al*., 2002). The acute challenge asthma

models successfully imitated the primary features of asthma i.e. AHR to specific stimuli, goblet cell hyperplasia, epithelial hypertrophy, elevated levels of IgE, airway inflammation. Moreover in some cases early and late phase broncho-constriction is also elicited. Nevertheless, there exist some main differences between the two. The pattern and distribution of pulmonary inflammation in acute asthma models is different from that observed in asthma patients. It is observed through histological analysis and broncho-alveolar lavage that the inflammatory cells present in the region are majorly eosinophils (Cho *et al*., 2004). The word "acute" refers to the short term response against the specific antigen and it has been observed that fundamental features of asthma like AHR and airway inflammation can resolve themselves within a period of few weeks after final allergen challenge. Due to its short term nature these models do not depict the chronic inflammation of airway walls and airway remodeling changes, which can be observed in chronic human asthma (McMillan and Lloyd, 2004).

Regardless of the above mentioned limitations, acute challenge models have proven its success in understanding the underlying mechanism of the disease. It has particularly accomplished understanding of the connection between the cells and inflammatory mediators and the mechanism by which these players initiate inflammatory response in lungs. The confirmation that the allergic response in asthma is Th-2 biased came from the studies in acute models. Moreover these models also made clear the role of eosinophil in the development of AHR and other clinical features of asthma (Foster *et al*., 1996). Many research groups are working on acute asthma models for improving the protocol and for developing a model that can be developed in short time and fulfill all the required characteristics. Many protocols are presented by research groups in which time period varies from 14 days to 32 days (Hamelmann *et al*., 1999). Siqueira *et al*. (1997), developed acute asthma models using different protocols and

compared the result with the other models showing pulmonary hypersensitity and based upon the results this group presents the appropriate murine model for asthma (de Siqueira *et al*., 1997).

Aravind *et al*. in his article discusses two protocol for acute allergen model. One protocol follows the antigen challenge via intra-tracheal route while another follow challenge by nebulization. Both the models successfully imitated the major characteristics of asthma. This group also suggested different ways of studying asthma, such as invasive as well as non-invasive techniques to study airway hyper responsiveness (Reddy *et al*., 2012). Daubeuf and Frossard during their research demonstrated the methods to competently achieve acute asthma features in mice using OVA as allergen. They discussed the parameters and methods capable of facilitating the group comparisons and assessment of potential of drug candidates (Daubeuf and Frossard, 2013).

Regardless of the success of acute asthma models, these models cannot be used in case of investigating the disease characteristics related to chronic asthma. Moreover these models will also show limitations during the assessment of potential novel treatments as its short term may hinder the actual results.

2.10.4 Chronic allergen challenge model:

Many groups have studied the chronic allergen exposure in mice in order to deal with the limitations associated with the acute challenge models. The main concern of these studies was to develop a model that can mimic additional features of clinical asthma so as to facilitate the research on potential novel drug therapies (Lloyd, 2007; Kumar *et al*., 2008).Chronic allergen models involve the repeated but low level of antigen exposure over an extended period of time as

compared to the acute models. This period can generally extend up to 12 weeks. In case of chronic models the adjuvant is not always required.

Temelkovski *et al*. showed that systemically sensitizing BALB/c mice with OVA and chronically challenging with OVA aerosols, resulted in the model capable of replicating many of the main features of clinical asthma. This study suggested that these models should assist in understanding of disease pathogenesis and potential therapeutic agents (Temelkovski *et al*., 1998). Another group also worked on OVA in order to create a chronic model. This group developed and compared the both acute and chronic asthma model and showed that chronic allergen model is more closely related to human asthma (Fernandez-Rodrigues *et al*., 2008). Another study showed that mechanism conducted by Th-2 cells and other inflammatory players, have significant role in airway inflammation as well as in airway remodeling (Wegmann, 2008). HDM extracts and grass pollen have also been investigated as allergen for inducing chronic asthma (Johnson *et al*., 2004; Kim *et al*., 2006). One of the main expected hurdle in the development of chronic asthma model, specifically using OVA is that continued exposure of the allergen may cause tolerance which will eventually lead to the suppression of the inflammatory response Jungsuwadee et al. Studies have shown that the tolerance is govern by the strain of the mouse used and the route of exposure. Moreover it is developed due to the high level of exposure of allergen (Shinagawa and Kojima, 2003; McMillan and Lloyd, 2004).

Chronic allergen models successfully duplicate the major characteristics of human asthma such as antigen-mediated sensitization, Th-2 biased inflammatory response with high invasion of inflammatory cells majorly eosinophils in mucosal lining of airways, and airway hyper-responsiveness. Most importantly studies have shown that some of the clinical features of asthma such as airway remodeling, persist even after termination of the allergen challenge(McMillan

and Lloyd, 2004). We cannot say that chronic challenge model is the perfect model as some limitations are also associated with these models. In some of the cases the characteristics of chronic asthma in mice are distinct from that of the humans. For instance, in humans inflammation is restricted to conducting airways where as in mice it is not (Wenzel and Holgate, 2006). Moreover, in mice mast cells are not recruited into the airway walls or epithelium (Boyce and Austen, 2005). The main characteristic of chronic asthma, the increase in the airway smooth muscle, does not appear in chronic allergen model (Wenzel and Holgate, 2006). A study showed that this feature can appear in some cases after a repeated and high level of antigen challenge (Shinagawa and Kojima, 2003).

Regardless of these limitations, the chronic allergen models have proved successful in mimicking significant characteristics of human asthma and the current studies are adding improvements in the protocols of chronic asthma models.

2.10.5 Assessing the "asthmatic" response

Considerable concerns occurs when assessing the results from the studies in OVA-challenge asthma models. These issues are related to the approaches and techniques employed for evaluation of response in models which cannot be corresponding to human assessment. Again at this point numerous alternatives are considered. So when studying the animal model different parameters are taken into account such as inflammation, airway hyper-responsiveness, airway remodeling, histology. All these parameters are studied for understanding the disease progression and characteristics.

2.10.5.1 Inflammation

One of the common method in assessing the level of inflammation is to quantify the total cell count and differential cell count in broncho-alveolar lavage (BAL) fluid. This experiment is quite suitable but one thing should be kept in mind that percentage increase of eosinophils in acute allergen challenge models is quite high as compared to the asthma patients. Cells obtained by BAL typically contain only 1-3% of the eosinophils where as in case of acute allergen models the percentage of eosinophils comprise up to 40 to 80% of the total cells (Fulkerson *et al*., 2005). On the other hand, chronic allergen asthmatic models do not show significant increase in BAL cells; only minor increase in the population of lymphocytes is seen and thus there exist a slight evidence of inflammatory cell recruitment (Kumar *et al*., 2002).

The studies have shown that the estimation of inflammatory response in tissue section is much more consistent. Tissue assessment show reliable information about the infilteration of eosinophils in airway epithelium as well as the accretion of the inflammatory cells, such as lymphocytes, plasma cells, macrophages, etc., in the lamina propria of the large airways. Both of these features can also be seen in typical human asthma (Bousquet *et al*., 1990). So studies show that the results can be compared between the model and human asthma we only have to look at the appropriate anatomical site. According to the literature it was found that in acute allergen models, even after a single challenge, eosinophils are readily detected in the airway epithelium of sensitized mice (Kumar *et al*., 2002). This phenomenon reflect the correspondence of tracheal epithelium in mice with bronchial epithelium in humans (Harkema, 1991).

2.10.5.2 Airway hyper-responsiveness

Airway hyper-responsiveness in mice can be measured both by invasive and non-invasive methods. A non-invasive measurement includes the exposure of the methacholine or histamine followed by the whole body plethysmography (Marin *et al*., 1988). In this method mice are placed in a plethysmography chamber for a certain period of time. The methacholine is given through nebulization and the mice are monitored for their respiratory variables for a certain period of time. Usually the monitoring cycle is of 6 minutes (Hamelmann *et al*., 1997).

The other invasive method relies on the tracheostomy. It is carried out on an anaesthetized ventilated mouse. Any which way, it is thought that AHR to methacholine in mice is related to the early airway closure due to thickening of the airways and inflammatory cells infiltration (Wagers *et al*., 2004). The measurement of AHR is highly influenced by the route of assessment i.e. if the cholinergic stimulus is given intravenously or via inhalation. Intravenous injections show noticeable response in OVA challenge models (Jonasson *et al*., 2009). Another method known as forced oscillation technique have proved to be successful in assessing the AHR. Tomioka *et al*., showed that this technique gives same results as obtained by alveolar capsule measured airway resistance. The results showed that this technique yield essential results even when the lungs are inhomogeneous. It gives a clear picture of bronchoconstriction in response to methacholine (Tomioka *et al*., 2002; Bates and Lutchen, 2005). There is another method which is recently introduced. It measures the decrease in tidal expiratory flow which can be done by both invasive and non-invasive method. This method can be performed repeatedly and hence gives repeatable AHR assessment (Glaab *et al*., 2005; Hoymann, 2007; Weckmann *et al*., 2007).

2.10.5.3 Airway remodeling

The main features of human asthma involve structural changes in airways like sub-epithelial fibrosis, smooth muscle hyperplasia or hypertrophy, increased vascularity of mucosa, etc. OVA challenge models can imitate some of the main features of chronic asthma (Ohkawara *et al*., 1997). In case of chronic allergen challenge models, widespread parenchymal inflammation ncan be seen which results in fibrosis of intra-pulmonary airways. This condition may also refer to peri-bronchiolar scarring and can be commonly referred as airway fibrosis (Kenyon *et al*., 2003). Conversely, acute allergen challenge models show tracheal lesions that are similar to the traditional sub-epithelial fibrosis (Temelkovski *et al*., 1998). Whereas the hypertrophy of airway smooth muscles is only elicited in the chronic allergen challenge models (Shinagawa and Kojima, 2003).

2.10.5.4 Histopathology

In order to study the morphological changes, the standard protocol for lung fixation is followed. By following this protocol animal model is sacrificed and its lungs and trachea are used to make the slides (Olmez *et al*., 2009). These slides are then observed for the physical manifestation of microscopic changes that take place at the tissue level under light microscope. The main feature of this disease is the abnormal epithelium surrounding the airways and thickened sub-epithelial smooth muscle. Moreover in some cases mononuclear infiltration in peri-bronchial parenchymal areas can also be seen. While when observing under electron microscope the one can see more details like thickening of sub-epithelial smooth muscle and regular basement membrane and as well as goblet cells (Kips *et al*., 2003). When cross sectional area of lungs is observed, it can be seen that the regular structure of bronchioles is distorted in shape in case of disease models.

OVA induced asthma models show very irregular or distorted cross-sectional structure when compared with the healthy ones. Moreover, in case of OVA sensitized models peri-bronchial fibrosis can also be observed (Shi *et al*., 2014). When the slides of OVA model and healthy mice are compared, massive infiltration of inflammatory cells may be observed in OVA group, and one can easily identify the AHR (Lee *et al*., 2006; Wang *et al*., 2014).

2.11 TOXICITY TESTING OF VIROSOME

Virosomes are composed of phospholipids and viral coat proteins, when talking about phospholipids, these compounds are considered safe for pharmaceutical use after testing. On the other hand the viral coat proteins and other constituents added to increase stability or enhance the properties of virosome, give virosomes toxic potential, specifically in case of parenteral administration. Our study is limited to common *in vitro* screening tests and general outline of tests required for drug registration.

2.11.1 Toxicity related to phospholipids given orally

Both virosomes and liposomes contain phospholipids as major constituent. Studies show that phosphatidylcholine in pure form has been used for many years as the major active ingredient of drugs that are administered orally or intravenously (Parnham and Wetzig, 1993). The standard toxicology investigation has shown that phospholipids are not related to any kind of acute or chronic forms of toxicity, as well as these are not related to mutagenicity and teratogenicity. But there exist certain limit i.e. phosphatidylcholine (PC) is tolerated upto 18 grams in daily intake. If the mentioned limit is exceeded it will result in gastro-intestinal discomfort such as nausea, diarrhea, etc (Kidd, 1996). Moreover PC is also associated with increased plasma cholesterol level if given for several weeks in high doses.

2.11.2. *In vitro* testing

In general *in vitro* tests are inexpensive and provide the results rapidly in comparison to in-vivo studies. Non- specialized laboratories can also perform these tests. *In vitro* testing is done in order to gain the understanding of basic mechanism of action of any component. *In vitro* testing may include several types of tests such as erythrocyte hemolysis tests and platelet aggregation test.

In hemolysis assay the human blood is mixed with test material in is incubated at the certain conditions of pH and temperature (the conditions that mimic cellular environment). This step is followed by the quantification of percent of red blood cells relative to the positive control. The desired result is negligible (Evans *et al*., 2013). Other test is platelet aggregation test, in which the test sample is added with the blood and is observed for the aggregation phenomenon. The aggregation is not favorable as it may lead to the development of thrombosis (Mesquita *et al*., 2013). This test is important if we are considering giving virosome intravenously but in this study the suggested route of administration is enteral route.

In vitro studies can also be carried out on cell cultures and human cell lines came out to be insensitive to liposomes. While when these studies were conducted on L1210 mouse leukemia cells toxic effects were observed. It was suggested that the phospholipid content has a little effect on cell growth and lipid composition of cell. Moreover to lesser extent it has an effect on dicetylphosphate and phosphatidic acid which are thought to be responsible for cell growth inhibitory effect (Panzer and Jansons, 1979; Layton *et al*., 1980; Campbell, 1983). While many others can be added, such as assessing the pyrogenicity and phagocytic index of the administered formulation and assessment of the tolerability after repeated administration.

2.11.3 *In vivo* testing

In vivo testing of virosomes can be carried out in mice, rats, guinea pigs, etc. All the procedures should be carried out according to the guidelines available for use of animals in testing. There are variety of ways by which animal can be used for testing such as the labeled virosome can be used to investigate distribution in the body by *in vivo* fluorescence imaging. This experiment was done using labeled liposome in rats. Pharmacokinetic as well as pharmacodynamic studies can also be done using an animal model. When a formulation is administered intranasally its concentration and deposition can also be assessed in lungs and other parts of an animal body. Moreover the blood of an animal can be analyzed for the possible effects caused by the administration of the particular formulation. Even the blood contents of the animal can be studied using HPLC or UPLC. Chen *et al*., (2012) successfully developed drug encapsulation liposome and studied their toxicity as well as the properties in rats and guinea pigs. This group was successful in proving that enclosing a drug in liposome causes increase retention in lungs and increase the therapeutic effect (Chen *et al*., 2012).

Chapter 3

MATERIALS AND METHODS

3.1 DRUG LOADED VIROSOMAL CONSTITUTION

3.1.1 Virus Sample

Influenza virus samples were obtained from Poultry Research Institute (PRI), Rawalpindi. This government institute is known to provide therapeutic and diagnostic expertise to poultry farmers of different districts of Punjab. In a routine process the birds exhibiting symptoms characteristics of influenza like illness are brought to the research institute for diagnosis. Only the birds exhibiting clinical signs and postmortem lesions of influenza are considered for sampling. The blood sample of 1 ml is collected from the wing vein of bird and Haemagglutination assays are carried out in order to ascertain the viral titer. Extracted samples contains diverse strains of avian influenza virus (AIV) for example H5, H7 and H9. The positive samples are further processed so that the strains can be sub-typed. After classifying the positive samples of virus according to the haemagglutinin proteins, the samples are further refined to determine the neuraminidase sub-type, using PCR. The samples are then inoculated in eight day old embryonated chicken eggs in order to escalate the viral titer. After the attainment of required viral titer i.e. one that could yield an MOI of 1, the allantoic fluid harboring the virus is obtained for further processing. The AIV samples are then distributed into aliquots, each measuring 250 µl, in 1.5ml microfuge tubes (Eppendorf, Germany), in class II Bio-safety Cabinet at Poultry Research Institute, Rawalpindi.

The aliquots are than washed with 3% phenol so that the virus can be incapacitated prior to further handling.

The aliquots containing the viral sample of H9N2 were brought from PRI to ASAB-NUST in dry ice- in order to maintain the viability of AIV - and stored at -80^{o}C.

3.1.2 Virus Egg Inoculation and Harvesting

Prior to egg inoculation, the live virus frozen at -80^{o}C was first thawed and syringe filtered with the filter of 0.2 μm pore size. Cell cultures and embryonated chicken eggs are commonly used for the propagation of these obligate intracellular parasites - viruses. For the growth of influenza virus, embryonated chicken eggs are commonly used. Woodruff, Goodpasture and Burnet in 1930, were the first to verify the use of eggs for the propagation of viruses (Burnet, 1936). The protocol established by these researchers is used till date for both the purpose of vaccine development and increasing viral titer. The current study also follows the same protocol.

Before inoculation of virus, the fertile eggs, brought from PRI, were kept at 38^{o}C, in humid environment in an incubator (INB 200, Memmert, Germany) and daily turned at 180^{o}. The eggs were checked daily for the viability by candling, the process which used special candling lamp containing incandescent bulb. Infertile eggs, showing dead embryos were discarded. Suitable sites of inoculation are selected according to the virus to be propagated. In case of AIV, both amnionic and amniotic fluid can be selected for inoculation. The current study targets the amniotic fluid as the potential site for virus inoculation. The area of chorioallantoic membrane was selected through candling. The blunt end of egg was held against the lamp and the air space was marked and the position of head of the embryo was also noted. The point of inoculation was marked a quarter away from the head and approximately 2 to 3 mm above the base of the air sac.

This point should be away from the embryo and amnionic cavity and should be devoid of the large blood vessels.

After determining the inoculation site of the viable cells, more than 100 eggs were selected for further processing. Next, the complete eggs, especially the inoculation sites, were disinfected using cotton swab dipped in 70% ethanol and pyodine. A small hole was pierced at the selected site with great care so as to avoid breakage to the egg membrane. A syringe of 1ml was used to introduce the virus inoculums of 200 µl into the allantoic fluid. The syringe was passed through the hole vertically; with the prediction that it will reach the allantoic fluid. This process was done slowly in order to avoid the spillage of the inoculum. Next, the opening was sealed using candle wax. The eggs after inoculation were kept in incubator at 37^{o}C in dark for 48 to 72 hours. After this period of incubation the eggs were chilled at 4^{o}C for at least 2 hours in order to kill the embryo and to avoid the chances of contamination of the allantoic fluid with blood during harvesting. Next the injection site was again disinfected. Sterile forceps were used to break the top of the shell. The allontoic fluid was harvested using syringe, being careful not to suck up yolk or blood. The harvested fluid was then kept in a sterile container and stored at -20^{o}C till further use. The spot test was carried out in order to confirm the presence of virus. Before carrying out spot test the virus harvest was first centrifuged at 10,000 rpm at 4^{o}C and the supernatant was then filtered through 0.22 µm syringe filter.

3.1.3 Virus Purification & Inactivation

With an aim to maintain the infectivity of virus particles, a method explained by Ueba was followed (Ueba, 1978). Samples showing positive spot were selected for further processing. Virus particles were removed from the egg harvest by PEG precipitation method. Virus

suspension of 500 µl was taken in microfuge tubes (Eppendorf) and same amount of 20% PEG was also added. The resulting mixture was then centrifuged at 20,000 rpm at 4^{o}C temperature for 45 minutes. After centrifugation the supernatant was discarded and the pellet containing virus particles was suspended in NT-buffer- 20% sucrose. Again the centrifugation was carried out and the pellet was obtained. The pellet was again resuspended and centrifuged through NTbuffer-30% sucrose. Again the pellet obtained was resuspended in NT-buffer-20% sucrose. Next the purified virus particles were inactivated by treatment with formaldehyde. The suspension was then pelleted by ultracentrifugation.

3.1.4 Virosome Constitution

Influenza virosome reconstitution was carried out following the protocol optimized during my BS thesis. The success of the protocol was assessed by different characterization studies such as Atomic Force Microscopy (AFM), Scanning Electron Microscopy (SEM), Bradford Protein Assay and Agarose Gel Electrophoresis. The reconstitution method was based on the reverse phase evaporation method. The process proceeds with the dissolution of 1.5g of lecithin in 10ml of diethyl ether (DEE) in order to make a solution of 0.02mM. Laterally, 1 mg of drug (sulbutamol sulfate) was added in distilled water. Next, 100 µl of influenza virus suspension (containing the extracted viral protein) along with 100 µl of 0.1 M NaCl and 100 µl of 0.01 M tris-HCl solution was added in this drug solution. The resulting solution was vortexed to complete the dissociation process. Lecithin and DEE solution were gradually injected into the aqueous suspension in the ratio of 1:1. The phase separation was formed between the two solutions. The resultant suspension was then mixed. The suspension was then given 5 cycles of sonication with amplitude of 0.5 nm and time period of 30 seconds. The process was repeated until the solution turned milky.

Based on the characterization studies done during the research of BS thesis, this method proved to yield a vehicle with high aqueous space-to-lipid ratio and increased potential to encapsulate drug present in the aqueous medium. The resultant carrier will have a neutral charge with a size distribution ranging from 30 to 500nm with the protein content approximately 0.5mg/ml.

3.1.5 Drug Entrapment Efficiency

The encapsulation efficiency of Salbutamol Sulfate (SBS) containing influenza virosome was assessed by the process depicted by Kumar et al, with slight modifications (Kumar *et al*., 2010). In concise, at first, the absorbance of precise 100µl of original virosomal suspension was measured and recorded at 273nm wavelength with spectrophotometer. Then 1.6ml of distilled water was added in the sample, followed by the addition of 90% (v/v) isopropyl alcohol containing 0.75M HCl to attain the final volume of 10 ml. The resultant solution was then centrifuged at 2000 rpm for 10 minutes. After that the supernatant was discarded. This process was repeated 3 times. The absorbance of treated sample was then measured and recorded by spectrophotometer at the wavelength of 273nm. Next, the drug entrapment efficiency was calculated by putting the values in the following formula

$$\text{Entrapment efficiency E.E (\%)} = A(v) / A(tot) \text{ X } 100$$

Where,

A(v) is the absorbance of the suspension after processing, and

A(tot) is the absorbance of the suspension without centrifugation.

3.1.6 Drug Release Assay

The drug release profile of the virosomal-SBS formulation was done *in vitro* by the dialysis bag method as mentioned by Shazly *at el*. with slight modifications (Shazly *et al.*, 2008). The process includes the assembly consisting of 15 ml and 50 ml falcon tubes. The aliquot of 0.5ml of virosomal formulation was taken in a small falcon tube (15 ml). The mouth of this falcon was sealed with the cellulose membrane filter paper with pore size of 0.22 μm so that the formation may not escape the falcon freely. This sealed falcon was then attached to the large (50 ml) falcon tube containing 30 ml of PBS in an inverted position such that its filter portion is slightly in contact with the PBS surface. This whole assembly was kept at 37^{o}C temperature. The pH of PBS was maintained at 7.4. Aliquots measuring 250 μl were sampled and the total volume of the solution was kept constant by adding same amount of fresh PBS. Absorbance of sample taken at regular intervals was calculated by spectrophotometer at 273 nm wavelength.

3.2 Safety Testing Of Virosome

Toxicity screening of virosome was done in-vivo by following the method described by Parnhum and Wetzig. For this purpose a group of eight female *BALB*/*c* mice were taken from the ASAB animal house lab. These mice were given the dose of 10 μl of virosome intra-tracheally. These mice were monitored for 48 hours for apparent symptoms of toxicity. Two mice from the group were sacrificed after regular intervals i.e. after 1, 4, 24 and 48 hours. The tissue samples from liver, lungs, kidneys and spleen were collected for histopathology analysis. During these experiments the weights of the mice were also recorded (Parnhum and Wetzig, 1993).

3.3 Evaluation Of Therapeutic Profile Of Drug Containing Virosome

For the purpose of evaluating the therapeutic profile of the drug loaded virosome an experiment was designed which was divided into two sections. First was the development of the asthmatic model in *BALB*/*c* mice and then the treatment of these mice with drug, drug containing virosome and liposomes. The basis of the experiment was taken from the article by Chen *et al*., who tested the efficacy of the liposome on asthmatic rats.

3.3.1 Development of Asthma Model

Asthmatic mouse model in *BALB*/*c* mice was developed on the basis of the protocol describe by Reddy *et al*.,2012). The protocol was optimized and altered according to the results and the materials present at ASAB-NUST. This protocol can be divided into two steps. First step is the sensitization of the mice and the other is the challenge of the sensitized mice with the antigen. For the development of asthma model 30 female *BALB*/*c* mice between the age of 6 to 8 weeks were taken from the ASAB animal house lab.

3.3.1.1 Sensitization of Mice

Sensitization of the mice was carried out using ovalbumin with alum as an adjuvant. For sensitization, the female *BALB*/*c* mice (6 to 8 weeks old) were given the intraperitoneal injection 0.15ml of the formulation. The formulation comprises of 20μg of ovalbumin and 2 mg of alum emulsified in 0.2ml of phosphate buffered saline. The sensitization process was completed in three weeks which comprised of three intraperitoneal injections of the formulation given on day 0, 7 and 14.

3.3.1.2 Challenge of Mice with Antigen

According to the modified protocol, the challenge step covers a whole week. The sensitized mice were given 3 days intratracheal challenge i.e. on day 18, 19 and 20, with 0.2% ovalbumin in PBS. These mice were then given inhalation challenge of 20 minutes with 0.2% ovalbumin in PBS for the next two days. The apparatus consists of a chamber which has a capacity to enclose more than three mice. This chamber comprises of two openings; one opening is connected with the falcon tube. This falcon receives the air pressure from the motor. The other end of the chamber takes air out into the motor. In this way air is circulated between the motor and the chamber. The air coming from the motor contains the droplets of the OVA solution. In this way 20 minutes inhalation challenge is given to the mice.

3.3.2 Confirmatory Tests for Asthma

After carrying out the above mentioned protocol these mice were tested for the confirmation of asthma. On the basis of literature review the mice models were examined physically for the symptoms and certain tests were also included in the study. These tests include complete blood count, differential leukocyte count, injection of β-blockers and challenge with cigarette smoke.

3.3.2.1 Physiological Changes in Mice

The animals were physically examined for symptoms such as lethargy, irregular breathing, redness around the nose and weight loss.

3.3.2.2 Differential Leukocyte Count

According to literature, the mice which developed asthma would show increase in the number of lymphocytes and eosinophills (Lacoste *et al*., 1993) . For that purpose 2 groups of 3 mice each

were taken representing healthy and diseased group respectively. These mice were sacrificed and 1 ml of blood was drawn from the heart of each mouse of the group. The blood sample of each mouse of both the diseased and healthy groups was divided into two EDTA tubes. One of the tubes was used for differential leukocyte count test from ASAB diagnostic laboratory and the other one was sent to the external diagnostic laboratory for eosinophill count test.

3.3.2.3 Beta Blockers

On the basis of literature it was known that normally β-blockers are avoided in asthma patients because these can trigger severe asthma attacks (Toogood, 1987). On the basis of this observation, two groups of healthy and diseased mice were injected with 0.2 ml of β-blocker (Labetalol) and were monitored for adverse effects.

3.3.2.4 Challenge with Cigarette Smoke

This experiment was designed on the basis of the study that cigarette smoke will trigger severe asthma attack by causing broncho-constriction (Moerloose *et al*., 2005). This experiment was carried out in specialized chamber as mentioned in the --- section. The suction pipe was connected with the cigarette and the motor was directly connected to the chamber. This resulted in the accumulation of cigarette smoke inside the chamber. There was one opening which allowed the smoke to escape the chamber. Each mouse was enclosed inside the chamber one by one and its pre-convulsion time was recorded i.e. the time from the start of the exposure till the induction of seizures. This experiment was done with both the healthy and diseased mice.

3.3.3 Evaluation of the Efficacy of Drug Containing Influenza Virosome

For evaluation of the efficacy of drug encapsulated influenza virosome, asthmatic mice were divided into different groups. The following table shows the scheme of division of the twenty diseased mice and five healthy mice which were further used in order to investigate the efficacy of the drug containing virosomes.

Table 3.1: Table representing the grouping of mice

Groups	**No of mice**	**Treatment**
Group I (Diseased group)	5	Virosome containing drug (SBS)
Group II (Diseased group)	5	Liposome containing drug (SBS)
Group III (Diseased Group)	5	Free drug
Group IV (Healthy Group)	5	For Reference
Group V (Disease Group)	5	For Reference

All of these mice were given the treatment after 48 hours of the last allergen challenge. The pre-convulsion time with cigarette smoke, of mice from each group was measured after regular

intervals in order to assess the level of broncho-constriction. Moreover, mouse from each group was sacrificed at 1 hour, 4 hours, 24 hours and 48 hours after the treatment. The lung tissues were collected from each mouse and further processed for the histological examination.

3.4 Tissue Processing For Histological Analysis

3.4.1 Formalin fixation

After sacrificing the mice the tissue samples of the lungs are immediately taken. A small tissue segment, approximately of about 0.5 to 1 cm^3 was taken from each sample. The obtained tissue samples of the lungs were then washed with dilute phosphate buffered saline (PBS) in order to wash away the red blood cells. Followed by the washing step, these tissue samples were than stored in neutral buffer formalin for minimum of 48 hours, in order to maintain the tissue morphology of the samples and to avoid any postmortem changes in the protoplast. The tissue samples can be stored in neutral buffer formalin for an indefinite period of time. Formalin is usually used to fix the tissue sample; this step will also facilitate the proper staining in the future.

3.4.2 Tissue processing

The formalin fixed tissues were first washed with distilled water in order to remove the fixative. Washing of the sample is followed by the process of dehydration which consists of the specimen treatment with the ascending concentration gradient of ethanol. After the dehydration step the dehydrating agent was removed by treatment of the specimen with xylene. Xylene is the clearing agent of choice as it is miscible with both dehydrating agent as well as embedding material. This step was then followed by addition of the melted paraffin wax in the sample containing falcon

and overnight incubation at 58^{o}C. The following table shows the scheme adopted for the tissue processing step by step with the duration for incubation.

Table 3.2: Table representing the process of tissue processing.

Step	**Process**	**Duration**
Tissue resection:	Lung tissue obtained from the mice	
Fixation	Place tissue segment in neutral buffer formalin	From 48 hours to indefinite period of time
Tissue Dehydration	70% ethanol	1 hour or overnight
	90% ethanol	1 hours
	100% ethanol	1 hour
	100% ethanol	1 hour
Clearing (Removal of dehydrating agent)	Xylene	1.5 hours
Infilteration:	Paraffin wax	Overnight incubation at 58^{o}C.

3.4.3 Tissue embedding:

Tissues after overnight incubation in paraffin wax were then allowed to solidify in the mold. In order to form a cube shaped block of paraffin containing tissue section, a tissue was placed on a mold and the mold was filled with paraffin. At first, the paraffin was allowed to solidify at room

temperature and after some time, the cassettes were then placed at 4^{o}C. These cassettes were stored at 4^{o}C prior to their usage for sectioning.

3.4.4 Sectioning:

Sectioning is done by an apparatus called microtome which is present in Neuro-biology lab at ASAB. The prepared blocks were then sliced by microtome into sections of 5-6 um thinness. These sections were obtained in the form of ribbons. These ribbons were then allowed to float on the water with maintained temperature of 40^{o}C. These sections were then mounted on the glass slides. Then these slides were dried by incubation at 37^{o}C for at least 20 min. This step was followed by the hematoxylin and eosin (H&E) staining.

3.4.5 Hematoxylin and Eosin (H&E) staining

Table 3.3: Table showing the protocol followed during H&E staining of the samples.

Step	Chemical	Duration
De-paraffinization:	Xylene I	3-5 minutes
	Xylene II	2-3 minutes
Rehydration	100% ethanol	3 minutes
	90% ethanol	1 minute
	80% ethanol	1 minute
	70% ethanol	1 minute
	Distilled water	4 to 5 dips
Hematoxylin staining	Hematoxylin	5 to 15 minutes
	D. water	3-5 dips
Counter staining	Eosin	2 to 5 seconds
	D. water	3-5 dips
Dehydration	70% ethanol	2 dips
	90% ethanol	2 dips
	100% ethanol	2 dips

3.4.6 Microscopic examination of the stained slides:

The prepared slides were then observed under the microscope with the magnification of 40X and 100X. Slides were observed for different histopathological changes like hemorrhages, necrosis, abnormal epithelium, signs of inflammation or structural alteration in bronchi and bronchioles.

Chapter 4

RESULTS

4.1 VIRUS INFECTION CONFIRMATION

A total of 100 embryonated chicken eggs (6-9 days old) were inoculated with the virus inoculums, obtained from poultry research institute (PRI). The virus inoculums of 200 µl were injected into the allantoic fluid of embryonated eggs. After incubation for 48 to 72 hours the eggs were observed for the signs of infection and were placed at -20^{o}C in order to kill the embryo. Death of the embryos was confirmed by visual observation. Confirmation of death of embryo was not only necessary for identifying the propagation of the virus but also conform to the biosafety SOPs set out. Figure 4.1 represents the dead chicken embryo in the egg.

Figure 4.1: An 11 day old embryonated chicken egg showing a dead embryo after virus inoculation and cold temperature treatment.

4.2 SPOT TEST FOR VIRUS CONFIRMATION

After processing the virus harvest obtained from the eggs, spot test was performed for each sample for confirmation of the virus propagation. Only the harvests showing high agglutination in spot test were selected for additional purification so that the harvest having high viral titer can be used further for the virosomal preparation.

4.3 DRUG ENTRAPMENT EFFICIENCY

As explained in chapter 3 for calculating drug entrapment efficiency, two aliquots from the virosome suspension were taken. One of them was added with Isopropyl alcohol (IPA) and Hydrochloric acid (HCl). Three measurements of absorbance were taken for both, processed and unprocessed, suspensions. The observations are reported in Table 4.1. The percentage drug entrapment efficiency was calculated thereafter by using a formula.

Table 4.1: Absorbance values of treated and untreated virosome sample determined for Drug Entrapment Efficiency.

Sr No	**Absorbance of washed/ treated virosome suspension**	**Absorbance of untreated virosome suspension**
1	0.923	1.10
2	0.931	1.05
3	0.926	1.12
Mean Value	**0.926**	**1.09**

By putting the mean values in the following formula the mean percentage drug entrapment efficiency was calculated to be 84.95 %.

$$\text{Entrapment efficiency E.E (\%)} = A_{(v)} / A_{(tot)} \text{ X } 100$$

$$\text{E.E\%} = 0.926/1.09 \text{ X } 100 = 84.95\%$$

4.4 DRUG RELEASE EFFICIENCY

Drug release efficiency was calculated by dialysis method with modifications. The method employs measuring the optical density of PBS samples taken at regular intervals. PBS samples were taken every hour for the first 12 hours; then the samples were taken after every 24 hours for 96 hours. Table 4.2 and Figure 4.2 depict the *in vitro* release profile of salbutamol sulphate from the Influenza virosome.

Table 4.2: Absorbance values of PBS samples taken after regular intervals

Time (Hours)	**Absorbance**
1	0.110
2	0.120
3	0.209
4	0.191
5	0.163
6	0.141
7	0.137
12	0.124
24	0.109
48	0.112
72	0.104
96	0.099

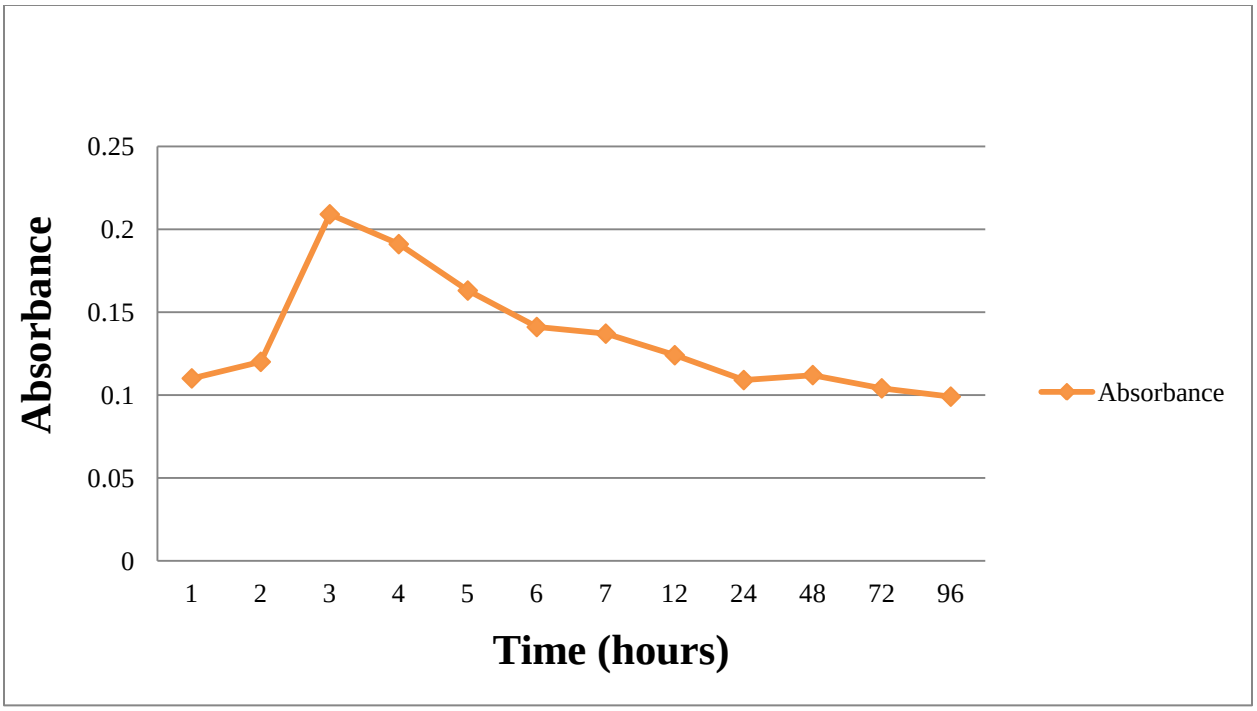

Figure 4.2: The graphical representation of absorbance values of PBS samples containing salbutamol sulphate, taken at regular intervals as mentioned in Table 4.2.

4.5 SAFETY TESTING OF DRUG ENCAPSULATED INFLUENZA VIROSOME (*IN VIVO)*

The toxicity of virosomes was investigated by intra-tracheal administration of virosome formulation in mice and the changes were observed by histological analysis. These animals were monitored for 48 hours and no apparent symptoms of toxicity were seen. Histological analysis of the slides from liver, lungs, kidneys and spleen, showed no signs of toxicity as shown in figures 4.3, 4.4, 4.5 and 4.6 respectively.

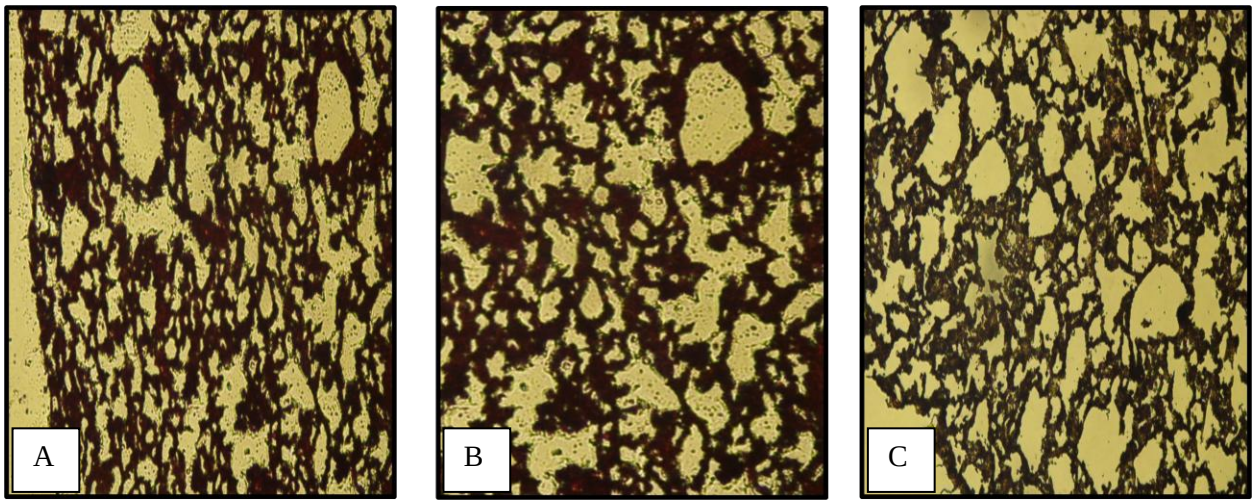

Figure 4.3: Histopathology of lungs at 40X (A) Image showing the histology of lungs of the healthy mice. (B) Histology image of mice lungs given virosome formulation after 24 hours and (C) 48 hours.

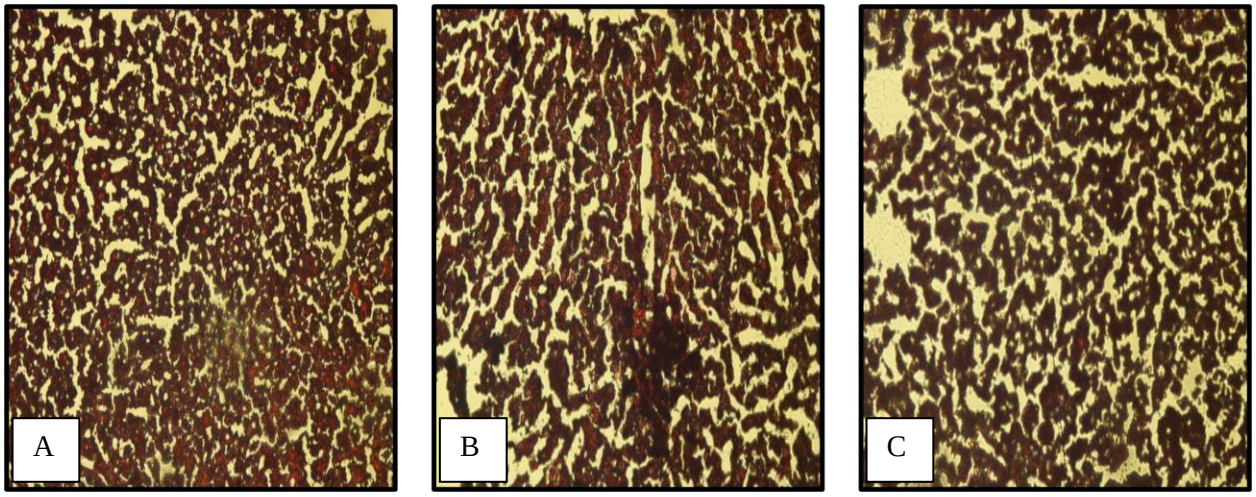

Figure 4.4: Histopathology of liver at 40X (A) Image showing the histology of liver of the healthy mice. (B) Histology image of mice liver given virosome formulation after 24 hours and (C) 48 hours.

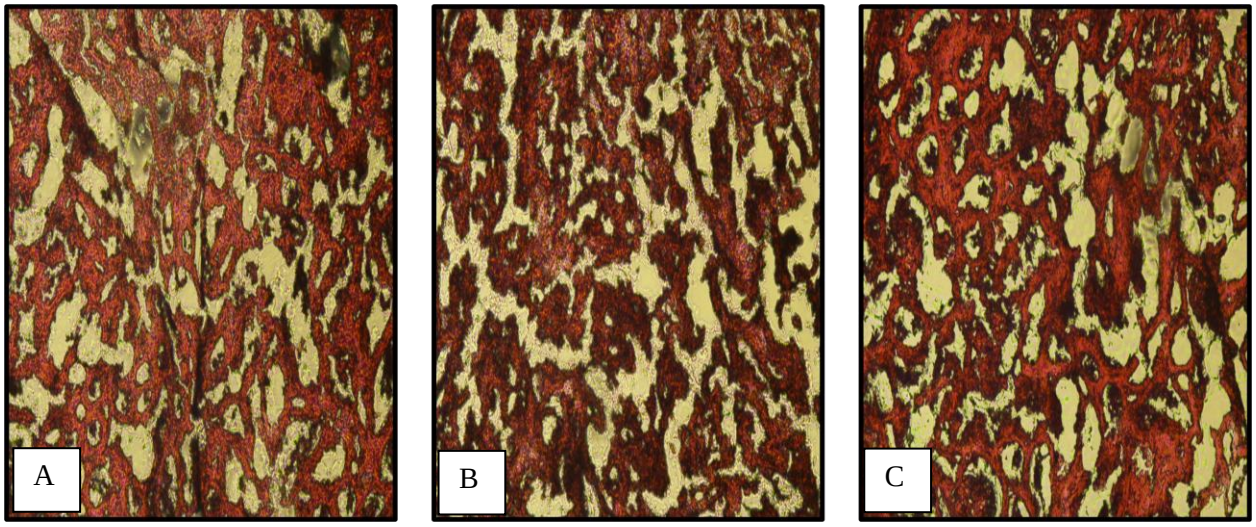

Figure 4.5: Histopathology of kidneys at 40X (A) Image showing the histology of kidneys of the healthy mice. (B) Histology image of mice kidneys given virosome formulation after 24 hours and (C) 48 hours.

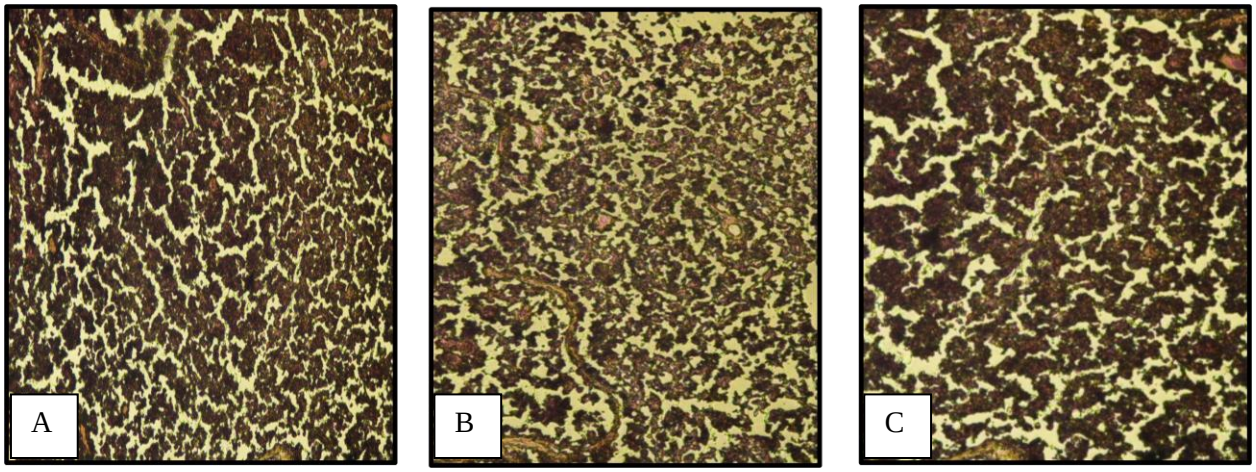

Figure 4.6: Histopathology of spleen at 40X (A) Image showing the histology of spleen of the healthy mice. (B) Histology image of mice spleen given virosome formulation after 24 hours and (C) 48 hours.

4.5.1 Analyzing Weight Trends during Toxicity Testing

Weekly body weight gain of control and trial group was recorded 3 weeks before the treatment and continued for 3 weeks after the treatment. It was seen that group of mice given virosome doses for toxicity testing followed the normal trend i.e. increase in a regular fashion. The following table shows the average body weights recorded for both the healthy and diseased groups for over a period of 6 weeks.

Table 4.3: Average body weights recorded for healthy and diseased groups

Week	**Healthy**	**V.toxiciy**
1	32.4	31.4
2	33.8	33.1
3	34.6	33.8
4	36.4	35.7
5	35.9	35.6
6	36	36

The above mentioned data can be interpreted with the help of the following graph which was drawn by employing the statistical analysis tool, Microsoft Excel.

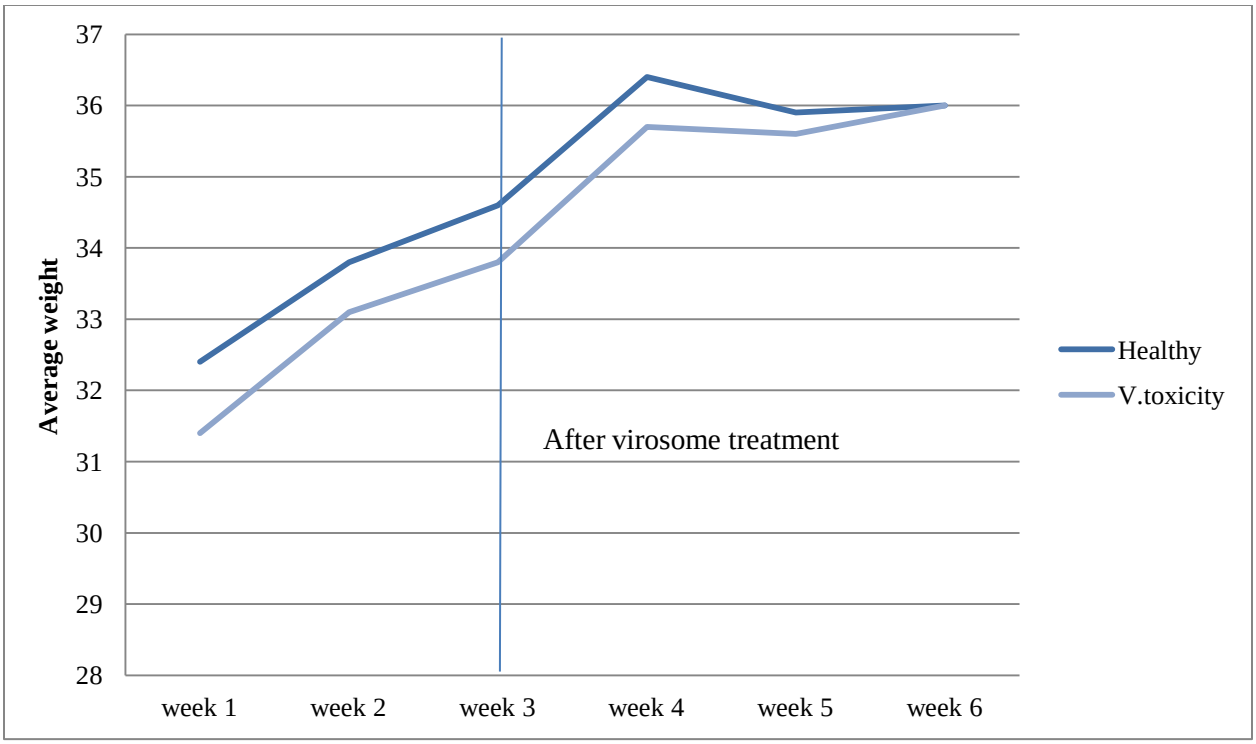

Figure 4.7: Graph showing comparison of weight between healthy and virosome treated mice. It is the graphical representation of data of table, where x-axis represents the weeks and y-axis represents average weights of mice.

They were no apparent signs of toxicity seen during physical examination of mice. Moreover, the histology images of the lungs, liver, spleen and kidneys showed no difference when compared with the healthy tissue slides.

4.6 CONFIRMATORY TESTS FOR ASTHMA IN DEVELOPED ASTHMA MODELS

4.6.1 Physiological Changes in Mice

After sacrificing the healthy and asthmatic mice, their lungs were observed for apparent disease signs. Hemorrhages were observed on the lungs of some (not all) of the mice with developed asthma symptoms (Figure 4.8).

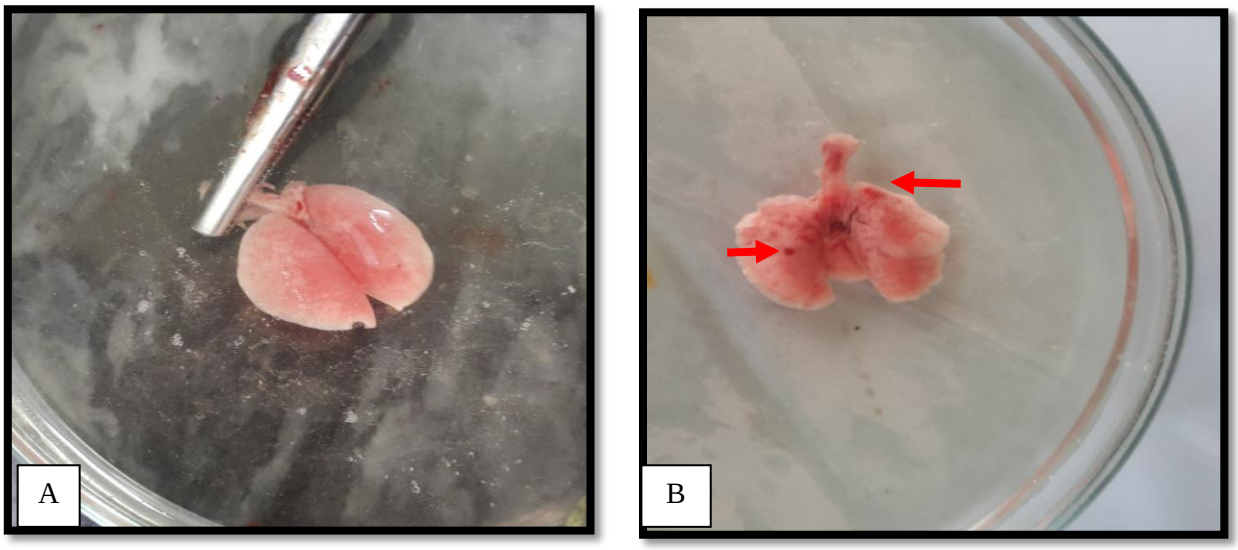

Figure 4.8: (A) Showing the lungs of a healthy mouse. (B) Showing the lungs of asthmatic mouse where the red arrows pointing the hemorrhages on the lungs.

Redness around there nose and irregular breathing was observed in all the mice with developed asthma.

4.6.2 Differential Leukocyte Count

As mentioned previously, it was expected that the asthmatic model would have increased amount of eosinophil and lymphocyte levels in blood. So the mice blood was tested for Differential Leukocyte Count and Eosinophil Count.

It was found that there exist a slight difference in the average values of eosinophil count (EC) in healthy and diseased mice. The average EC of healthy group was calculated to be 108/µl while the diseased group gave the average of 126/µl of the blood. The results of this observation are represented in the figure 4.9.

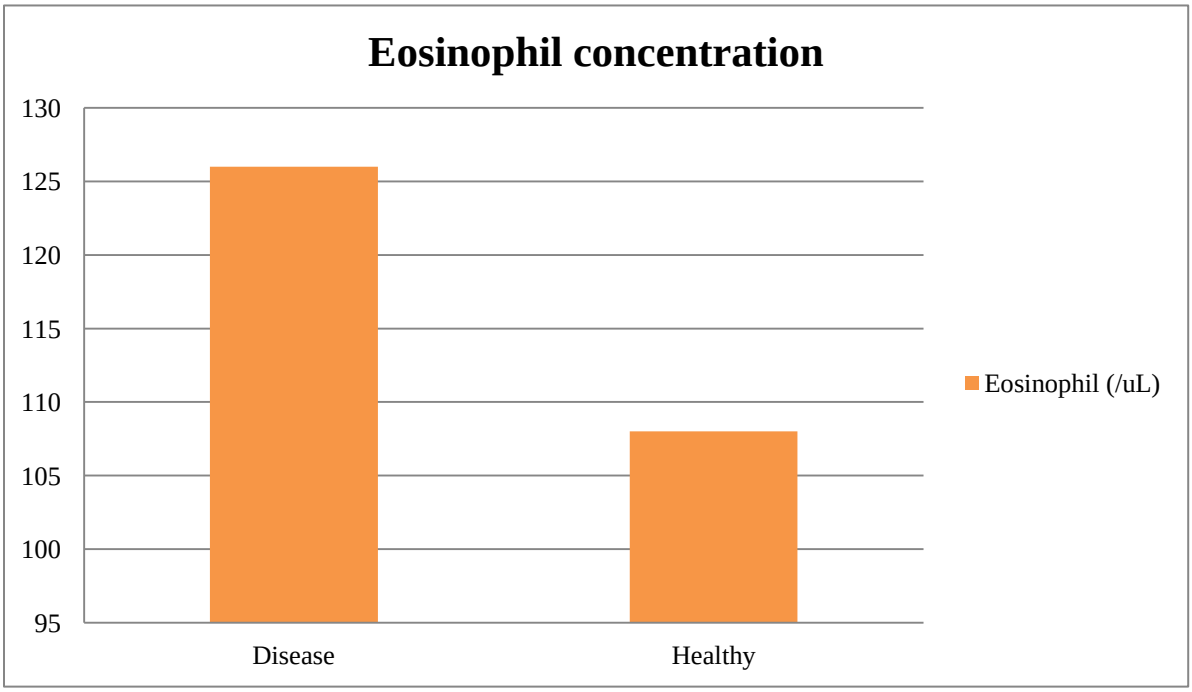

Figure 4.9: The graphical representation of the comparison of eosinophil count of healthy and diseased mice per µl of blood.

Table 4.4: Table representing the average values of concentration of WBCs and lymphocytes calculated in healthy and diseased groups.

	Healthy mice	**Diseased mice**
Lymphocytes	3.9K / µl	7.3 K/ µl
White Blood Cells	4.9K/ µl	9.94K/ µl

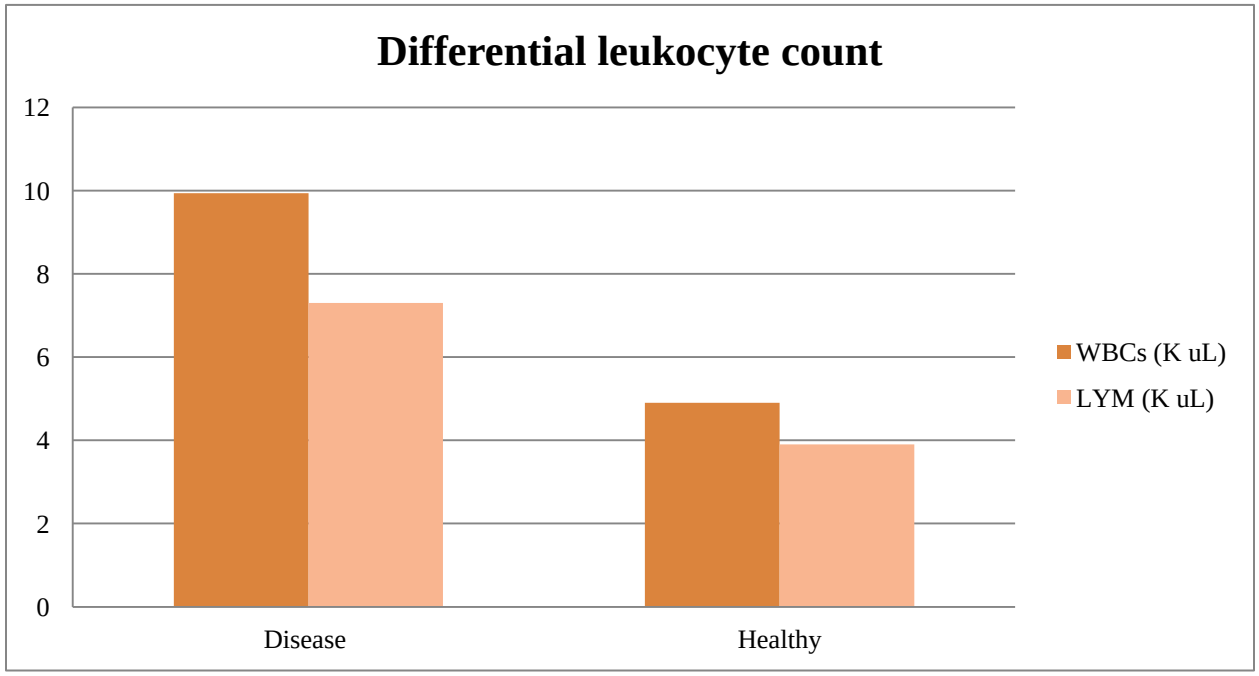

Figure 4.10: The graphical representation of the comparison of concentration of WBCs and lymphocytes of healthy and diseased mice per µl of blood.

4.6.3 Injection of β -Blockers

Beta blockers generally aren't used in people with asthma because of concerns that the medication may trigger severe asthma attacks. The injection of β -blocker (Labetalol) resulted in irregular breathing, wheezing, sneezing and seizers. These effects were observed in the diseased group, while the healthy mice were not affected at all by the 0.2ml injection.

4.6.4 Challenge with The Antigen

In this case cigarette smoke was used as the challenge in order to trigger broncho-constriction in the asthmatic mice. Cigarette smoke was filled in the chamber and the pre-convulsion time (time period in which the mouse start having seizers) of 10 mice were calculated. On average the preconvulsion time for both the diseased and healthy mice was calculated to be 35.75 seconds and 2.5 minutes respectively.

4.6.5 Comparison between the Histology slides of Healthy and Diseased Group

The histology slides of healthy and diseased groups were compared in order to find the difference. These slides were also compared with reference slides present in literature and the certain areas of problem were highlighted (Figure 4.11).

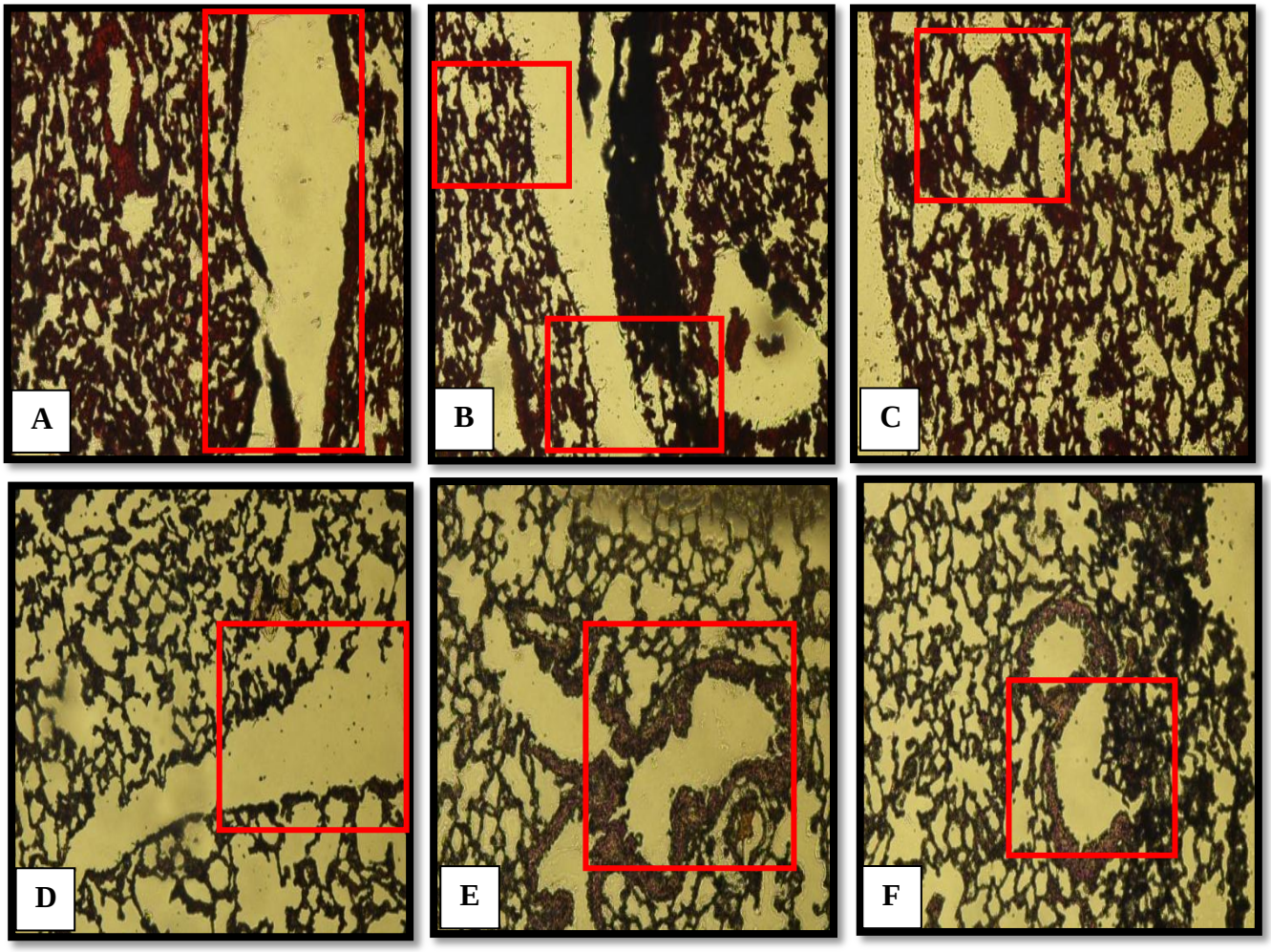

Figure 4.11: (A), (B), (C) representing slides of lungs obtained from healthy mice while (D),(E),(F) representing slides of lungs from the diseased mice; where the areas of concern are highlighted with red boxes. (A) Transverse section of bronchioles showing the healthy walls as marked by the red shape. (B) Normal air spaces between the lung tissues. (C) Cross sectional area of bronchioles. (D) Damaged walls of bronchioles. (E) & (F) Increased air spaces and distorted shape of bronchioles.

4.7 EVALUATION OF THE DISEASE CONDITION AFTER TREATMENT

4.7.1 Calculation of Pre-Convulsion Time

The pre- convulsion time for each group was again calculated after 24 & 48 hours of treatment with free drug, drug containing liposomes and drug containing virosomes. At 24 hours all the groups showed considerable increase in pre-convulsion time as compared to the time recorded before treatment. Whereas the pre-convulsion time remain extended after 48 hours in case of the groups given virosome and liposome treatment. The results of this observation are depicted in the following table (Table 4.5).

Table 4.5: Average pre-convulsion time period calculated before and after the treatments

	Pre-convulsion time		
	Before treatment	**After treatment**	
		24 hours	**48 hours**
Free drug	Average period of the diseased group 35.75 seconds	1.30 minutes	58 seconds
Liposome		1.06 minutes	1.16 minutes
Virosome		1.25 minutes	1.30 minutes

4.7.2 Trends in Weight After The Treatment

The average increase or decrease in weight was recorded for all the 5 groups of mice representing diseased, healthy and the other groups that were given the treatment of virosome, liposome and free drug. Table 4.6 shows the weekly weight records for all the groups.

Table 4.5: Variations in the weight recorded over the period of 6 weeks

Week	Group I (Disease)	Group II (Liposome)	Group III (Virosome)	Group IV (Drug)	Group V (Healthy)
1	32.8	32.4	35.2	29.6	32.4
2	35	32.8	32.4	32.4	33.8
3	35.4	34	33.7	36.5	34.6
4	34.6	30	30.1	32	36.4
5	27.25	27.3	25.4	30.6	35.9
6	30.2	29.9	27.48	3.9	36

Sensitization

Challenge step

After treatment

4.7.3 Assessment Of The Physiological Changes

Animals from each group were sacrificed at predetermined time intervals after the treatment. The lung tissues were collected from each mouse and were further processed for the histological examination as represented in figures 4.12, 4.13 and 4.14.

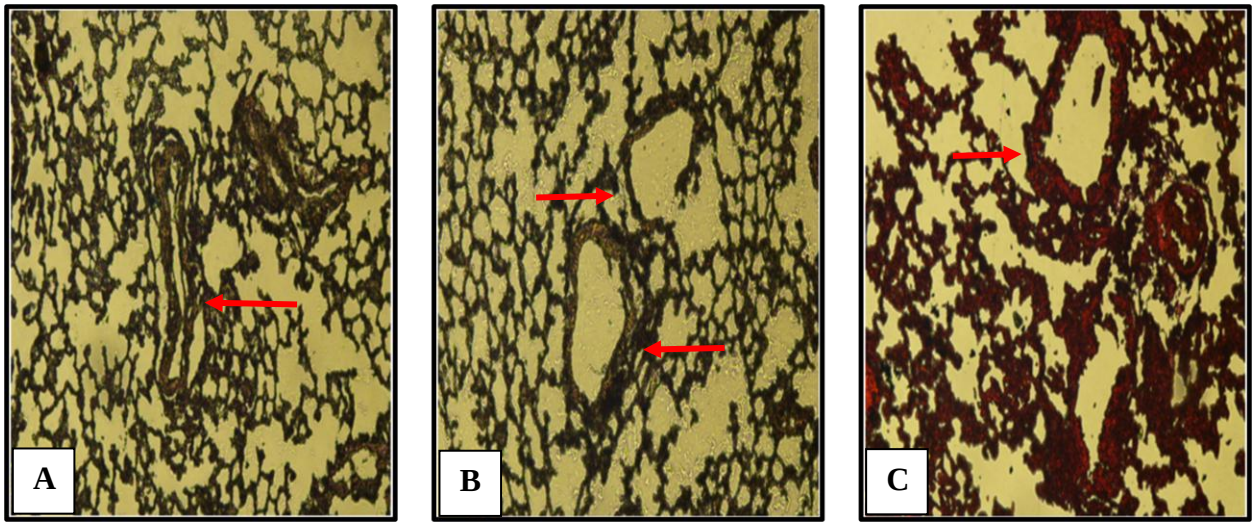

Figure 4.12: Histology slides obtain from the lungs after the treatment of free drug at (A) 4 hours, (B) 24 hours and (C) 48 hours, respectively. The changes in the shape of the bronchioles are highlighted with red arrows.

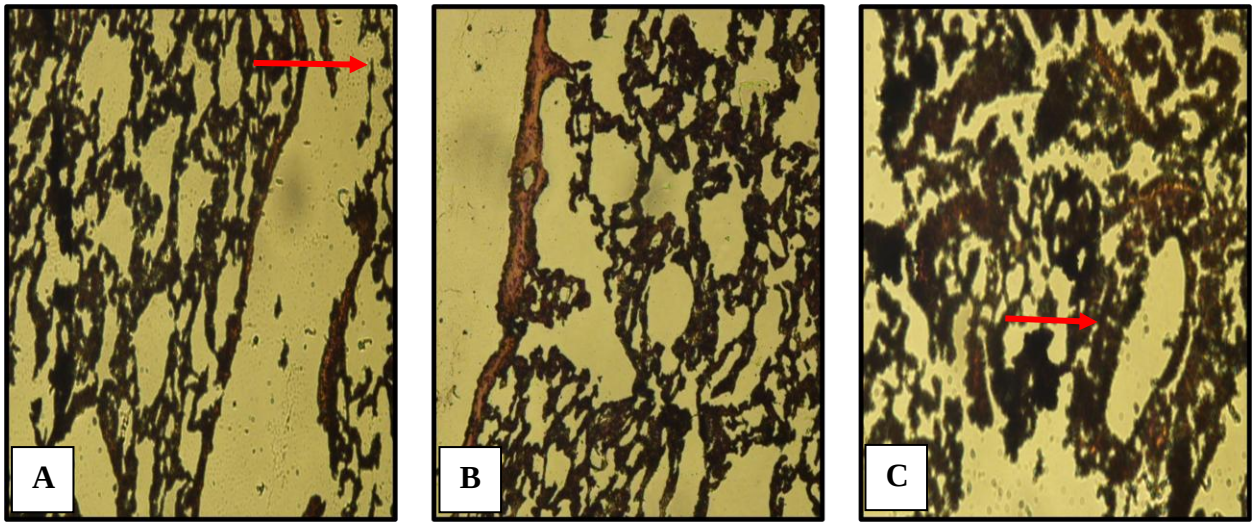

Figure 4.13: Histology slides obtain from the lungs after the treatment of liposome containing drug at (A) 4 hours, showing the increased airspaces and the damaged wall of bronchi (B) 24 hours and (C) 48 hours, respectively.

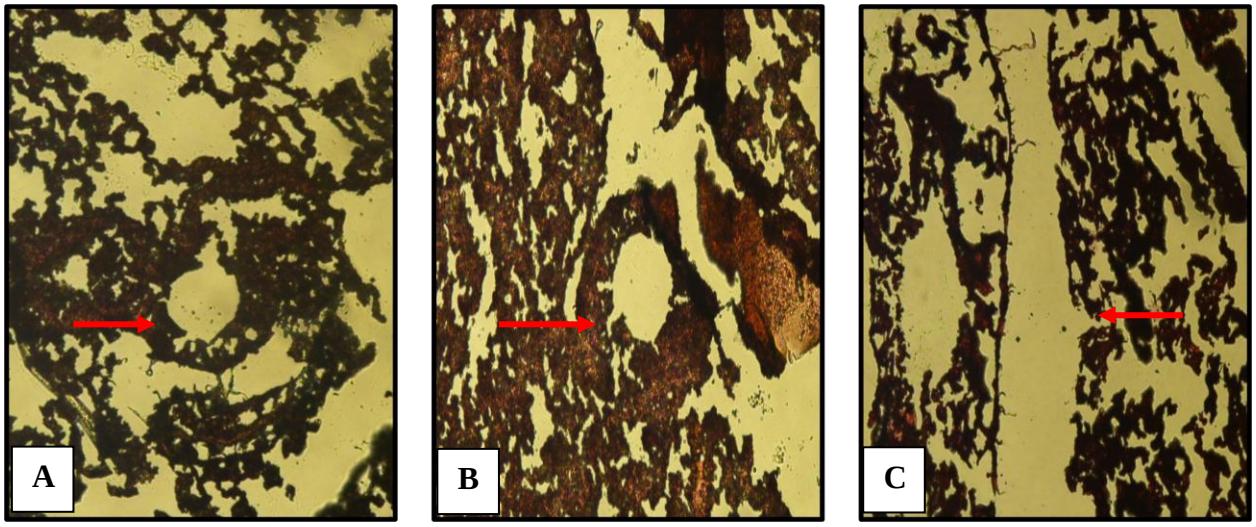

Figure 4.14: Histology slides obtain from the lungs after the treatment of virosome containing drug at (A) 4 hours, (B) 24 hours and (C) 48 hours, respectively. The cross-sectional and transverse sectional image of bronchioles.

Chapter 5

DISCUSSION

Virosomes are the reconstituted viral envelopes devoid of their genetic material and consisting of membrane phospholipids and glycoproteins. These are so constructed that their pathogenicity is eliminated while retaining their fusogenic activity (Kalra *et al*., 2013). So far different viruses have been reconstituted successfully including rabies, Semliki Virus, Vesicular Stomatitis Virus, Hepatitis B, influenza virus, etc. (Helenius *et al*., 1981; Perrin *et al*., 1985; Metsikko *et al*., 1986). During the recent years, virosome technology have successfully broaden its application in different fields of biology as vaccines, adjuvants and delivering agents (Saravanan *et al*., 1986) . Different virosomal products have entered the market such as Epaxal and Inflexal, which are used for vaccination purpose against Hepatitis A virus and Influenza virus respectively (Mischler *et al*., 2002; Bovier, 2008), while several others are going through clinical and pre-clinical trials.

The present study consisted of reconstitution of Influenza virus encapsulating drug and evaluation of its safety and its therapeutic profile in asthmatic model. The first is related to the constitution of virosome and second part is related to the development of an asthma model. In the present study, sufficient viral titer was attained by egg inoculation method owing to easy availability of embryonated chicken eggs and their capability to raise the viral titer efficiently. The virus obtained by egg inoculation was first inactivated by phenol before further use in order to reduce the chance of infection during further handling.

As mentioned in the previous chapters the virosome was produced by using the protocol optimized during our previous research. The method includes the disruption of viral envelope by

addition of detergents and rearrangement of lipids and surface glycoproteins after removal of the detergent (Bron, 1993). After reviewing literature and series of experiment related to reconstitution and characterization a protocol was finalized with the best and constant results. In our study, we used 0.1 M NaCl and 0.01 M tris-HCl solution as a detergent. Tris HCl acts as a buffer and also plays an important role in lysis of the virus. This mixture has been known to lyse the virus and release the viral surface glycoproteins, HA and NA. The treatment of the samples with these chemicals was followed by the centrifugation in order to separate viral samples with debris. This process confirmed the attainment of relatively purer quantities of viral protein.

Virosome prepared by the traditional methods was approximately 150 nm in size (Abdoli, 2013). According to the results of the previous research, this method generates the virosomes ranging in size from 30 to 700 nm. On the basis of the results obtained by scanning electron microscope virosomes produced remained in the range of 30 to 100 nm. An average size below 100nm confirms the efficiency of the process developed and followed. Different methods employed for the reconstitution of the viruses produce virosomes of different sizes (Sarachai, 2013). In the present study the drug encapsulated, salbutamol sulphate, was water soluble, so there was no need to add few drops of NaOH in the virosomal formulation. As this formulation was to be used *in vivo* so this chemical would have its additional toxic effects in mice. The other chemical which was used and expected to be harmful was diethyl ether, but no apparent toxic effects were seen.

Drug entrapment efficiency was calculated by treating the virosome suspension with IPA and HCl in order to disrupt the vesicles. By taking three consecutive readings and using their mean value in the formula, the entrapment efficiency of virosome was calculated to be 84.95%. Nature of the drug i.e. hydrophilicity or lipophilicity, determines the Entrapment Efficiency of the drug

(Shazly *et al*., 2008). In literature, a range of EEs can be seen for different drugs such as sinomenine-liposome showing the drug EE of 64.9%, Nimesulide-liposome showing the maximum of EE of 83.92% and Gentifinib-liposome showing the excellent EE of about 92.9% (Zhou *et al*., 2006; Kumar *et al*., 2010; Hea *et al*., 2012). In our own previous study, sulfasalazine was encapsulated with entrapment efficiency of 89%.

Drug release efficiency was carried out by following the dialysis method. Virosomes containing the drug molecules were subjected to interact with PBS. The sample of PBS was taken after regular intervals for measuring absorbance. The readings of which were represented in the form of line graph. The analysis of the line graph shows that the rate of drug release increases abruptly during the first three hours and reaches to the maximum at the third hour and then decreases gradually. The samples taken after 24 hours showed marked decrease in the rate and the minimum rate of drug release was obtained at the 96th hour. The trend could help in establishing the controlled release properties of the preparation.

Asthmatic models have an elementary place in asthma research. Owing to the complex and diverse nature of this disease, it is highly unlikely that a single mouse model can be regarded as fully representative of the disease. Usually each model is developed in order to mimic specific phenotype of asthma. This research focuses on the development of acute allergen challenge models because of the required characteristics i.e. airway hyper-responsiveness and pulmonary inflammation.

In order to test the toxicity of the virosome the mice were injected intra-tracheally by virosome formulation. Intra-tracheal is the suggested route of administration, so the toxicity testing was done using this route. These animals were monitored for 48 hours and no apparent symptoms of

toxicity were observed. Histological analysis of the slides from liver, lungs, kidneys and spleen, showed no sign of toxicity. Weight changes also followed the normal trends i.e. increase up to certain age and then decrease with time.

After thorough literature review, different tests were found that could be used for confirmation of asthma. Still many were short listed according to the facilities and expertise available. Differential leukocyte count and eosinophil count tests, performed on the blood gave considerable results but if these tests would have been performed on Broncho-alveolar lavage, the results would have been better. The tail-vein injection of β-blocker resulted in seizures in the diseased mice whereas the healthy mice remained un-affected. This experiment was tried on the basis of the information that the β-blocker causes broncho-constriction in asthma patients and was successful. Similarly, cigarette smoke was successfully used as an agent in order to elicit bronco-constriction and for the calculation of the convulsion time. In literature this experiment is usually done using methacholine and histamine. In the current research, we also tried to use saw dust as allergen but that did not work. After confirmation of the asthma development the protocol was further optimized to get better results. It was seen that the sensitized mice which were given antigen challenge through intra-tracheal route developed asthma earlier and showed more visible symptoms.

When analyzing the histology slides the cross-sectional as well as the transverse sectional image of the bronchioles were noted. In asthma the walls of bronchioles become damaged and the cross-sectional round shape of the bronchioles also become distorted. Moreover the air spaces increase in case of asthma.

When trends in weight for the treated groups were recorded , a gradual increase in weight was seen till sensitization period. By analyzing the graph a very slight decrease in weight was seen after the exposure challenge in all the 4 diseased groups. In normal trend it is known that weight of mice increases with age. But in diseased models it was seen that during the challenge period the weight starts to decrease followed by the rapid decrease in 5 weeks. While analyzing the lines representing liposome, drug & virosome showed that the weight increased after 24 to 48 hours of treatment.

By assessing the weight trends, pre-convulsion time and histology images it was found that the virosome showed comparable results with free drug and liposomes. The pre-convulsion time for the virosome treated group showed the prolonged effect of the drug.

Conclusion and Future Prospects

Influenza Virosome was successfully formed with entrapment of Salbutamol sulphate drug, showing 84.95 % entrapment efficiency and sustained release up to 48 hours. Favorable results of toxicity testing were obtained, as virosomes showed no sign of toxicity. The drug release efficiency analysis showed that the drug levels were sustained in the formulation for up to 48 hours. By assessing the weight trends, pre-convulsion time and histology images it was found that the virosome showed comparable results with free drug and liposomes. The ease of the preparation of virosomes withen capsulated drug and the favorable results related to the toxicity and efficacy, provides a positive opportunity for increasing the half live and bioavailability of different drugs.

Virosomes have numerous applications including their use as targeted drug delivery agents for neurodegenerative diseases and cancers .They can also be used as agents for vaccinations. Moreover, virosomes act as delivery vehicles for genetic materials. In addition, virosomes can be used to increase the half life of different drugs with short half life. Further research is needed in the field of virosomal technology.

Chapter 6

REFERENCES

Abdol.A., S. H. (2013). Determining Influenza Virus Shedding at Different Time Points in Madin-Darby Canine Kidney Cell Line. Cell Journal, 15(2): 130-135.

Abdoli, A., Soleimanjahi, H., Kheiri, M. T., Jamali, A., Sohani, H., Abdoli, M., & Rahmatollahi, H. R. (2013). Reconstruction of H3N2 influenza virus based virosome in-vitro. Iranian journal of microbiology, 5(2): 166.

Adamina, M., Guller, U., Bracci, L., Heberer, M., Spagnoli, G. C., & Schumacher, R. (2006). Clinical applications of virosomes in cancer immunotherapy. Expert opinion on biological therapy, 6(11): 1113-1121.

Almeida, J., Edwards, D. C., Brand, C., & Heath, T. (1975). Formation of virosomes from influenza subunits and liposomes. The Lancet, 306(7941): 899-901.

Alvarez, J., Surs, W., Leung, D. Y., Iklé, D., Gelfand, E. W., & Szefler, S. J. (1992). Steroid-resistant asthma: immunologic and pharmacologic features. Journal of allergy and clinical immunology, 89(3): 714-721.

Arkema, A., Huckriede, A., Schoen, P., Wilschut, J., & Daemen, T. (2000). Induction of cytotoxic T lymphocyte activity by fusion-active peptide-containing virosomes. Vaccine, 18(14): 1327-1333.

Asher, M. I., Montefort, S., Björkstén, B., Lai, C. K., Strachan, D. P., Weiland, S. K., Phase Three Study Group. (2006). Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. The Lancet, 368(9537): 733-743.

Babar, M. M., Kazi, A. G., & Rehman, A. (2014). Virosomes-Hybrid Drug Delivery Systems. Journal of Antivirals & Antiretrovirals, 2013.

Bagai, S., & Sarkar, D. P. (1993). Reconstituted Sendai virus envelopes as biological carriers: dual role of F protein in binding and fusion with liver cells.Biochimica et Biophysica Acta (BBA)-Biomembranes, 1152(1): 15-25.

Barrett, E. G., Rudolph, K., Bowen, L. E., Muggenburg, B. A., & Bice, D. E. (2003). Effect of inhaled ultrafine carbon particles on the allergic airway response in ragweed-sensitized dogs. Inhalation toxicology, 15(2): 151-165.

Bartlett, N. W., Walton, R. P., Edwards, M. R., Aniscenko, J., Caramori, G., Zhu, J., ... & Tuthill, T. J. (2008). Mouse models of rhinovirus-induced disease and exacerbation of allergic airway inflammation. Nature medicine,14(2): 199-204.

Bates, J. H., & Lutchen, K. R. (2005). The interface between measurement and modeling of peripheral lung mechanics. Respiratory physiology & neurobiology, 148(1): 153-164.

Bautsch, W., Hoymann, H. G., Zhang, Q., Meier-Wiedenbach, I., Raschke, U., Ames, R. S., Klos, A. (2000). Cutting edge: guinea pigs with a natural C3a-receptor defect exhibit decreased bronchoconstriction in allergic airway disease: evidence for an involvement of the C3a anaphylatoxin in the pathogenesis of asthma. The Journal of Immunology, 165(10): 5401-5405.

Bice, D. E., Seagrave, J., & Green, F. H. (2000). Animal models of asthma: potential usefulness for studying health effects of inhaled particles. Inhalation toxicology, 12(9): 829-862.

Blease, K., Schuh, J. M., Jakubzick, C., Lukacs, N. W., Kunkel, S. L., Joshi, B. H., ... & Hogaboam, C. M. (2002). Stat6-deficient mice develop airway hyperresponsiveness and peribronchial fibrosis during chronic fungal asthma. The American journal of pathology, 160(2): 481-490.

Blyth, D. I., Pedrick, M. S., Savage, T. J., Hessel, E. M., & Fattah, D. (1996). Lung inflammation and epithelial changes in a murine model of atopic asthma. American journal of respiratory cell and molecular biology, 14(5): 425-438.

Boivin S, C. S. (2010). Influenza A virus polymerase: structural insights into replication and host adaptation mechanisms. The Journal of Biological Chemistry, 285(37): 28411-28417.

Bomsel, M., Tudor, D., Drillet, A. S., Alfsen, A., Ganor, Y., Roger, M. G., ... & Devillier, G. (2011). Immunization with HIV-1 gp41 subunit virosomes induces mucosal antibodies protecting nonhuman primates against vaginal SHIV challenges. Immunity, 34(2): 269-280.

Bousquet, J., Chanez, P., Lacoste, J. Y., Barnéon, G., Ghavanian, N., Enander, I., ... & Michel, F. B. (1990). Eosinophilic inflammation in asthma. New England Journal of Medicine, 323(15): 1033-1039.

Bovier, P. A. (2008). Epaxal®: a virosomal vaccine to prevent hepatitis A infection. Expert review of vaccines, 7(8): 1141-1150.

Boyce, J. A., & Austen, K. F. (2005). No audible wheezing nuggets and conundrums from mouse asthma models. The Journal of experimental medicine, 201(12): 1869-1873.

Bron, R., Ortiz, A., & Wilschut, J. (1994). Cellular cytoplasmic delivery of a polypeptide toxin by reconstituted influenza virus envelopes (virosomes).Biochemistry, 33(31): 9110-9117.

Bui, M., Whittaker, G., & Helenius, A. (1996). Effect of M1 protein and low pH on nuclear transport of influenza virus ribonucleoproteins. Journal of virology, 70(12): 8391-8401.

Bungener, L., de Mare, A., de Vries-Idema, J., Sehr, P., van der Zee, A., Wilschut, J., & Daemen, T. (2006). A virosomal immunization strategy against cervical cancer and pre-malignant cervical disease. Antiviral therapy,11(6): 717.

Campbell, P. I. (1982). Toxicity of some charged lipids used in liposome preparations. Cytobios, 37(145): 21-26.

Cech, P. G., Aebi, T., Abdallah, M. S., Mpina, M., Machunda, E. B., Westerfeld, N., ... & Daubenberger, C. (2011). Virosome-formulated Plasmodium falciparum AMA-1 & CSP derived peptides as malaria vaccine: randomized phase 1b trial in semi-immune adults & children. PloS one, 6(7): e22273.

Chapoval, S. P., Iijima, K., Marietta, E. V., Smart, M. K., Chapoval, A. I., Andrews, A. G., & David, C. S. (2002). Allergic inflammatory response to short ragweed allergenic extract in HLA-DQ transgenic mice lacking CD4 gene. The Journal of Immunology, 168(2): 890-899.

Chen, X., Huang, W., Wong, B. C., Yin, L., Wong, Y. F., Xu, M., & Yang, Z. (2012). Liposomes prolong the therapeutic effect of anti-asthmatic medication via pulmonary delivery. International journal of nanomedicine, 7: 1139.

Cho, J. Y., Miller, M., Baek, K. J., Han, J. W., Nayar, J., Lee, S. Y., ... & Broide, D. H. (2004). Inhibition of airway remodeling in IL-5–deficient mice. The Journal of clinical investigation, 113(4): 551-560.

Chopra, R. N., & Chopra, I. C. (1933). Indigenous drugs of India. Academic publishers.

Clarke, D. L., Davis, N. H., Majithiya, J. B., Piper, S. C., Lewis, A., Sleeman, M. A., Corkill D. J.& May, R. D. (2014). Development of a mouse model mimicking key aspects of a viral asthma exacerbation. Clinical Science,126(8): 567-580.

Cohen S., S. A. (2011). How viruses access the nucleus. . Biochimica et Biophysica Acta, 1813, 1634–1645.

Colasurdo, G. N., Hemming, V. G., Prince, G. A., Gelfand, A. S., Loader, J. E., & Larsen, G. L. (1998). Human respiratory syncytial virus produces prolonged alterations of neural control in airways of developing ferrets. American journal of respiratory and critical care medicine, 157(5): 1506-1511.

Connor, R. J., Kawaoka, Y., Webster, R. G., & Paulson, J. C. (1994). Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology, 205(1): 17-23.

Content J, W. L. (1977). Cell-free coupling of influenza virus RNA transcription and translation. Journal of Virology, 22(2): 247–255.

Cornet, B., Vandenbranden, M., Cogniaux, J., Giurgea, L., Dekegel, D., & Ruysschaert, J. M. (1990). Virosomes reconstituted from human immunodeficiency virus proteins and lipids. Biochemical and biophysical research communications, 167(1): 222-231.

Correale P1, C. M. (2001). Tumour-associated antigen (TAA)-specific cytotoxic T cell (CTL) response in vitro and in a mouse model, induced by TAA-plasmids delivered by influenza virosomes. 37(16).

Cusi, M. G. (2006). Applications of influenza virosomes as a delivery system. Human Vaccines, 2(1): 1-7.

Cusi, M. G., Zurbriggen, R., Valassina, M., Bianchi, S., Durrer, P., Valensin, P. E., ... & Glück, R. (2000). Intranasal immunization with mumps virus DNA vaccine delivered by influenza virosomes elicits mucosal and systemic immunity. Virology, 277(1): 111-118.

Daemen, T., de Mare, A., Bungener, L., de Jonge, J., Huckriede, A., & Wilschut, J. (2005). Virosomes for antigen and DNA delivery. Advanced drug delivery reviews, 57(3): 451-463.

Daubenberger, C. A., Pluschke, G., Zurbriggen, R., & Westerfeld, N. (2008). Development of influenza virosome-based synthetic malaria vaccines. Expert opinion on drug discovery, 3(4): 415-423.

Daubeuf, F., & Frossard, N. (2013). Acute asthma models to ovalbumin in the mouse. Current protocols in mouse biology, 31-37.

de Jonge, J., Leenhouts, J. M., Holtrop, M., Schoen, P., Scherrer, P., Cullis, P. R., ... & Huckriede, A. (2007). Cellular gene transfer mediated by influenza virosomes with encapsulated plasmid DNA. Biochemical Journal,405(1): 41-49.

de Jonge, J., Schoen, P., Stegmann, T., Wilschut, J., & Huckriede, A. (2006). Use of a dialyzable short-chain phospholipid for efficient solubilization and reconstitution of influenza virus envelopes. Biochimica Et Biophysica Acta (BBA)-Biomembranes, 1758(4): 527-536.

de Siqueira, A. L. P., Russo, M., Steil, A. A., Facincone, S., Mariano, M., & Jancar, S. (1997). A new murine model of pulmonary eosinophilic hypersensitivity: contribution to experimental asthma. Journal of allergy and clinical immunology, 100(3): 383-388.

Dias A, B. D. (2009). The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. . Nature 458: 914-918.

Eisenbarth, S. C., Piggott, D. A., Huleatt, J. W., Visintin, I., Herrick, C. A., & Bottomly, K. (2002). Lipopolysaccharide-enhanced, toll-like receptor 4–dependent T helper cell type 2 responses to inhaled antigen. The Journal of experimental medicine, 196(12): 1645-1651.

Evans, B. C., Nelson, C. E., Shann, S. Y., Beavers, K. R., Kim, A. J., Li, H., ... & Duvall, C. L. (2013). Ex vivo red blood cell hemolysis assay for the evaluation of pH-responsive endosomolytic agents for cytosolic delivery of biomacromolecular drugs. JoVE (Journal of Visualized Experiments), (73): e50166-e50166.

Ewart, S. L., Kuperman, D., Schadt, E., Tankersley, C., Grupe, A., Shubitowski, D. M., Peltz G.& Wills-Karp, M. (2000). Quantitative trait loci controlling allergen-induced airway hyperresponsiveness in inbred mice. American journal of respiratory cell and molecular biology, 23(4): 537-545.

Fairbairn, S. M., Page, C. P., Lees, P., & Cunningham, F. M. (1993). Early neutrophil but not eosinophil or platelet recruitment to the lungs of allergic horses following antigen exposure. Clinical & Experimental Allergy, 23(10): 821-828.

Felnerova, D., Viret, J. F., Glück, R., & Moser, C. (2004). Liposomes and virosomes as delivery systems for antigens, nucleic acids and drugs.Current Opinion in Biotechnology, 15(6): 518-529.

Fernandez-Rodriguez, S., Ford, W. R., Broadley, K. J., & Kidd, E. J. (2008). Establishing the phenotype in novel acute and chronic murine models of allergic asthma. International immunopharmacology, 8(5): 756-763.

Fishbein, A. B., Lee, T. A., Cai, M., Oh, S. S., Eng, C., Hu, D., Huntsman, S., Farber, H.J., Serebrisky, D., Silverberg, J. & Williams, L. K. (2016). Sensitization to mouse and cockroach allergens and asthma morbidity in urban minority youth: Genes-environments and Admixture in Latino American (GALA-II) and Study of African-Americans, Asthma, Genes, and Environments (SAGE-II). Annals of Allergy, Asthma & Immunology.

Fleddermann, J., Diamanti, E., Azinas, S., Košutić, M., Dähne, L., Estrela-Lopis, I, Moya, S. E. (2016). Virosome engineering of colloidal particles and surfaces: bioinspired fusion to supported lipid layers. Nanoscale, 8(15): 7933-7941.

Fodor E. (2013). The RNA polymerase of influenza a virus: mechanisms of viral transcription and replication. Acta Virologica. 57(2): 113-122.

Foster, P. S., Hogan, S. P., Ramsay, A. J., Matthaei, K. I., & Young, I. G. (1996). Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. The Journal of experimental medicine, 183(1): 195-201.

Foster, P. S., Yang, M., Herbert, C., & Kumar, R. K. (2002). CD4+ T-Lymphocytes Regulate Airway Remodeling and Hyper-Reactivity in a Mouse Model of Chronic Asthma. Laboratory investigation, 82(4): 455-462.

Fredberg, G. P., Herz, U., Inman, M. D., Jordana, M., Kemeny, D.M., Lötvall, J., Pauwels, R.A., Plopper, C.G. and Schmidt, D. (2003). Murine models of asthma. European Respiratory Journal, 22(2); 374-382.

Fuchs, B., & Braun, A. (2008). Improved mouse models of allergy and allergic asthma-chances beyond ovalbumin. Current drug targets, 9(6): 495-502.

Fulkerson, P. C., Rothenberg, M. E., & Hogan, S. P. (2005). Building a better mouse model: experimental models of chronic asthma. Clinical & Experimental Allergy, 35(10): 1251-1253.

Gambaryan AS1, T. A. (1997). Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6'-sialyl...,. Virology, 232(2): 345-350.

Gamblin, S. J., & Skehel, J. J. (2010). Influenza hemagglutinin and neuraminidase membrane glycoproteins. Journal of Biological Chemistry,285(37): 28403-28409.

Glaab, T., Ziegert, M., Baelder, R., Korolewitz, R., Braun, A., Hohlfeld, J. M., ... & Hoymann, H. G. (2005). Invasive versus noninvasive measurement of allergic and cholinergic airway responsiveness in mice. Respiratory research,6(1): 1.

Greenspan, D., Krystal, M., Nakada, S., Arnheiter, H., Lyles, D. S., & Palese, P. (1985). Expression of influenza virus NS2 nonstructural protein in bacteria and localization of NS2 in infected eucaryotic cells. Journal of virology, 54(3): 833-843.

Grimaldi, S., Giuliani, A., Ferroni, L., Lisi, A., Santoro, N., & Pozzi, D. (1995). Engineered liposomes and virosomes for delivery of macromolecules. Research in virology, 146(4): 289-293.

Guo CT1, T. N. (2007). The quail and chicken intestine have sialyl-galactose sugar chains responsible for the binding of influenza A viruses to human type receptors. Glycobiology, 17(7): 713-724.

Gürtler, L. (2006). Virology of human influenza. Influenza Report. Kamps BS, Hoffmann C, Preiser W. Flying Publisher, Wuppertal.

Hamelmann, E., Schwarze, J., Takeda, K., Oshiba, A., Larsen, G. L., Irvin, C. G., & Gelfand, E. W. (1997). Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography.American journal of respiratory and critical care medicine, 156(3): 766-775.

Hamelmann, E., Tadeda, K., Oshiba, A., & Gelfand, E. W. (1999). Role of IgE in the development of allergic airway inflammation and airway hyperresponsiveness–a murine model. Allergy, 54(4): 297-305.

Harkema, J. R. (1991). Comparative aspects of nasal airway anatomy: relevance to inhalation toxicology. Toxicologic pathology, 19(4-1): 321-336.

Helenius, A., Fries, E., & Kartenbeck, J. (1977). Reconstitution of Semliki forest virus membrane. The Journal of cell biology, 75(3): 866-880.

HELENIUS, A., SARVAS, M., & SIMONS, K. (1981). Asymmetric and symmetric membrane reconstitution by detergent elimination. European Journal of Biochemistry, 116(1): 27-35.

Hoymann, H. G. (2007). Invasive and noninvasive lung function measurements in rodents. Journal of pharmacological and toxicological methods, 55(1): 16-26.

Huckriede, A., Bungener, L., ter Veer, W., Holtrop, M., Daemen, T., Palache, A. M., & Wilschut, J. (2003). Influenza virosomes: combining optimal presentation of hemagglutinin with immunopotentiating activity. Vaccine,21(9): 925-931.

Huckriede, A., De Jonge, J., Holtrop, M., & Wilschut, J. (2007). Cellular delivery of siRNA mediated by fusion-active virosomes. Journal of liposome research, 17(1): 39-47.

Hunziker I P, G. B. (2002). In vitro studies of core peptide-bearing immunopotentiating reconstituted influenza virosomes as a non-live prototype vaccine against hepatitis C virus. . International Immunology 14 (6): 615-626.

Ito, K., Herbert, C., Siegle, J. S., Vuppusetty, C., Hansbro, N., Thomas, P. S., Kumar, R. K. (2008). Steroid-resistant neutrophilic inflammation in a mouse model of an acute exacerbation of asthma. American journal of respiratory cell and molecular biology, 39(5): 543-550.

Ito, T., & Kawaoka, Y. (2000). Host-range barrier of influenza A viruses.Veterinary microbiology, 74(1): 71-75.

Johnson, J. R., Wiley, R. E., Fattouh, R., Swirski, F. K., Gajewska, B. U., Coyle, A. J., Jordana, M. (2004). Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. American journal of respiratory and critical care medicine, 169(3): 378-385.

Jonasson, S., Hedenstierna, G., Hedenström, H., & Hjoberg, J. (2009). Comparisons of effects of intravenous and inhaled methacholine on airway physiology in a murine asthma model. Respiratory physiology & neurobiology, 165(2): 229-236.

Kalra, N., Dhanya, V., Saini, V., & Jeyabalan, G. (2013). Virosomes: As a Drug Delivery Carrier. American Journal of Advanced Drug Delivery, 1(1): 29-35.

Kamphuis, T. (2012). A virosomal respiratory syncytial virus vaccine candidate with a Toll-like receptor ligand as built-in adjuvant (Doctoral dissertation, University of Groningen).

Kaneda, Y. (2000). Virosomes: evolution of the liposome as a targeted drug delivery system. Advanced drug delivery reviews, 43(2): 197-205.

Kannan, R. M., PERUMAL, O. P., & Kannan, S. (2006). Cellular Interactions of Nano Drug Delivery Systems. FORCE MICROSCOPY, 113.

Karol, M. H. (1994). Animal models of occupational asthma. European Respiratory Journal, 7(3): 555-568.

Kawaguchi, Y., Miyamoto, Y., Inoue, T., & Kaneda, Y. (2009). Efficient eradication of hormone-resistant human prostate cancers by inactivated Sendai virus particle. International journal of cancer, 124(10): 2478-2487.

Keiko Sato, T. T. (2010). How to Classify Influenza A Viruses and Understand Their Severity. World Scientific Publishing Company, Vol. 17, No. 3: 297–310.

Kenyon, N. J., Ward, R. W., & Last, J. A. (2003). Airway fibrosis in a mouse model of airway inflammation. Toxicology and applied pharmacology, 186(2): 90-100.

Kidd, P. M. (1996). Phosphatidylcholine, a superior protectant against liver damage. Altern Med Rev, 1(4): 258-74.

Kim, C. H., Ahn, J. H., Kim, S. J., Lee, S. Y., Kim, Y. K., Kim, K. H., Kwon, S. S. (2006). Co-administration of vaccination with DNA encoding T cell epitope on the Der p and BCG inhibited airway remodeling in a murine model of chronic asthma. Journal of Asthma, 43(5): 345-353.

Kips, J. C., Anderson, G. P., Fredberg, J. J., Herz, U., Inman, M. D., Jordana, M., ... & Schmidt, D. (2003). Murine models of asthma. European Respiratory Journal, 22(2): 374-382.

Kumar, R. K., Herbert, C., & Foster, P. S. (2008). The “classical” ovalbumin challenge model of asthma in mice. Current drug targets, 9(6): 485-494.

Kumar, R. K., Herbert, C., Yang, M., Koskinen, A. M., McKenzie, A. N., & Foster, P. S. (2002). Role of interleukin-13 in eosinophil accumulation and airway remodelling in a mouse model of chronic asthma. Clinical & Experimental Allergy, 32(7): 1104-1111.

Kurup, V. P., Choi, H., Murali, P. S., Resnick, A., Fink, J. N., & Coffman, R. L. (1997). Role of particulate antigens of Aspergillus in murine eosinophilia.International archives of allergy and immunology, 112(3): 270-278.

Lacoste, J. Y., Bousquet, J., Chanez, P., Van Vyve, T., Simony-Lafontaine, J., Lequeu, N., ... & Godard, P. (1993). Eosinophilic and neutrophilic inflammation in asthma, chronic bronchitis, and chronic obstructive pulmonary disease. Journal of Allergy and Clinical Immunology, 92(4): 537-548.

Lakadamyali M, M. J. (2004). Endocytosis of influenza viruses. . Microbes Infection, 6(10): 929–936.

Lambert, A. L., Winsett, D. W., Costa, D. L., SELGRADE, M. K., & IAN GILMOUR, M. (1998). Transfer of allergic airway responses with serum and lymphocytes from rats sensitized to dust mite. American journal of respiratory and critical care medicine, 157(6): 1991-1999.

Langer. R. (1998). Drug delivery and targeting. Luo M. (2012). Influenza virus entry. Advances in Experimental Medicine and Biology, 726: 201-221.

Layton, D., Luckenbach, G. A., Andreesen, R., & Munder, P. G. (1980). The interaction of liposomes with cells: the relation of cell specific toxicity to lipid composition. European Journal of Cancer (1965), 16(12): 1529-1538.

Lee, Y. C., Kim, S. H., Seo, Y. B., Roh, S. S., & Lee, J. C. (2006). Inhibitory effects of Actinidia polygama extract and cyclosporine A on OVA-induced eosinophilia and bronchial hyperresponsiveness in a murine model of asthma. International immunopharmacology, 6(4): 703-713.

Li, B. W., Bruijn, M. J., Tindemans, I., Lukkes, M., KleinJan, A., Hoogsteden, H. C., Hendriks, R. W. (2016). T cells are necessary for ILC2 activation in house dust mite-induced allergic airway inflammation in mice.European journal of immunology, 46(6): 1392-1403.

Lloyd, C. M. (2007). Building better mouse models of asthma. Current allergy and asthma reports, 7(3): 231-236.

Lowell, F. C. (1990, May). Observations on Heaves-An Asthma-Like Syndrome in the Horse. In Allergy and Asthma Proceedings (Vol. 11, No. 3, p. 149). OceanSide Publications.

M. Gowtham, M. P. (2012). 'Virosome' A Novel Strategy for Delivery of Drugs and Targeting: An Overview. 1(5).

Mario Amacker, O. E. (2005). Peptide-loaded chimeric influenza virosomes for efficient in vivo induction of cytotoxic T cells. 17(6).

Marsh, M., Bolzau, E., White, J., & Helenius, A. (1983). Interactions of Semliki Forest virus spike glycoprotein rosettes and vesicles with cultured cells. The Journal of cell biology, 96(2): 455-461.

Martha I. Nelson, E. C. (2007). The evolution of epidemic influenza. . Nature Reviews Genetics 8: 196-205.

Martin, T. R., Gerard, N. P., Galli, S. J., & Drazen, J. M. (1988). Pulmonary responses to bronchoconstrictor agonists in the mouse. Journal of Applied Physiology, 64(6): 2318-2323.

Mastrobattista, E., Schoen, P., Wilschut, J., Crommelin, D. J., & Storm, G. (2001). Targeting influenza virosomes to ovarian carcinoma cells. FEBS letters, 509(1): 71-76.

Mastrobattista, E., Schoen, P., Wilschut, J., Crommelin, D. J., & Storm, G. (2001). Targeting influenza virosomes to ovarian carcinoma cells. FEBS letters, 509(1): 71-76.

Matrosovich, M., Herrler, G., & Klenk, H. D. (2013). Sialic acid receptors of viruses. In SialoGlyco Chemistry and Biology II (pp. 1-28). Springer International Publishing.

Matsuoka, Y., Matsumae, H., Katoh, M., Eisfeld, A. J., Neumann, G., Hase, T., ... & Watanabe, S. (2013). A comprehensive map of the influenza A virus replication cycle. BMC systems biology, 7(1):1.

McIntire, J. J., Umetsu, S. E., Akbari, O., Potter, M., Kuchroo, V. K., Barsh, G. S., Freeman GJ, Umetsu DT & DeKruyff, R. H. (2001). Identification of Tapr (an airway hyperreactivity regulatory locus) and the linked Tim gene family. Nature immunology, 2(12): 1109-1116.

McMillan, S. J., & Lloyd, C. M. (2004). Prolonged allergen challenge in mice leads to persistent airway remodelling. Clinical & Experimental Allergy, 34(3): 497-507.

McMillan, S. J., & Lloyd, C. M. (2004). Prolonged allergen challenge in mice leads to persistent airway remodelling. Clinical & Experimental Allergy, 34(3): 497-507.

Mesquita, H. L. D., Carvalho, G. R. D., Aarestrup, F. M., Corrêa, J. O. D. A., & Azevedo, M. R. A. (2013). Evaluation of platelet aggregation in the presence of antiphospholipid antibodies: anti-β2GP1 and anticardiolipin.Revista brasileira de reumatologia, 53(5): 400-404.

Metsikkö, K., van Meer, G., & Simons, K. (1986). Reconstitution of the fusogenic activity of vesicular stomatitis virus. The EMBO journal, 5(13): 3429.

Michoud, M. C., Pare, P. D., Boucher, R., & Hogg, J. C. (1978). Airway responses to histamine and methocholine in Ascaris suum-allergic rhesus monkeys. Journal of Applied Physiology, 45(6): 846-851.

Miller, A. D. (2003). The problem with cationic liposome/micelle-based non-viral vector systems for gene therapy. Current medicinal chemistry, 10(14): 1195-1211.

Mischler, R., & Metcalfe, I. C. (2002). Inflexal® V a trivalent virosome subunit influenza vaccine: production. Vaccine, 20: B17-B23.

Moerloose, K. B., Pauwels, R. A., & Joos, G. F. (2005). Short-term cigarette smoke exposure enhances allergic airway inflammation in mice. American journal of respiratory and critical care medicine, 172(2): 168-172.

Morokata, T., Ishikawa, J., Ida, K., & Yamada, T. (1999). C57BL/6 mice are more susceptible to antigen-induced pulmonary eosinophilia than BALB/c mice, irrespective of systemic T helper 1/T helper 2 responses. Immunology,98(3): 345-351.

Moser, C., Müller, M., Kaeser, M. D., Weydemann, U., & Amacker, M. (2013). Influenza virosomes as vaccine adjuvant and carrier system. Expert review of vaccines, 12(7): 779-791.

Mueller, M. S., Renard, A., Boato, F., Vogel, D., Naegeli, M., Zurbriggen, R. Pluschke, G. (2003). Induction of parasite growth-inhibitory antibodies by a virosomal formulation of a peptidomimetic of loop I from domain III of Plasmodium falciparum apical membrane antigen 1. Infection and immunity,71(8): 4749-4758.

Nayak DP, H. E. (2004). Assembly and budding of influenza virus. Virus Research 106(2): 147-165.

Nerome, K., Yoshioka, Y., Ishida, M., Okuma, K., Oka, T., Kataoka, T., ... & Oya, A. (1990). Development of a new type of influenza subunit vaccine made by muramyldipeptide-liposome: enhancement of humoral and cellular immune responses. Vaccine, 8(5): 503-509.

Noori, M., Ghorbani, S., Jamali, A., Shenagari, M., Hashemi, H., & Kheiri, M. T. (2011, January). Construction of influenza virosome from influenza A H1N1 PR8. In BMC Proceedings (Vol. 5, No. Suppl 1, p. P5). BioMed Central Ltd.

O'Hagan, D. T. (2007). New Generation Vaccine Adjuvants. Okitsu SL, S. O. (2007). A Virosomal Malaria Peptide Vaccine Elicits a Long-Lasting Sporozoite-Inhibitory Antibody Response in a Phase 1a Clinical Trial. 2(12).

Ohkawara, Y., Lei, X. F., Stämpfli, M. R., Marshall, J. S., Xing, Z., & Jordana, M. (1997). Cytokine and eosinophil responses in the lung, peripheral blood, and bone marrow compartments in a murine model of allergen-induced airways inflammation. American journal of respiratory cell and molecular biology, 16(5): 510-520.

Olmez, D., Babayigit, A., Erbil, G., Karaman, O., Bagriyanik, A., Yilmaz, O., & Uzuner, N. (2009). 6 Histopathologic Changes in Two Mouse Models of Asthma. Journal of investigational allergology & clinical immunology, 19(2): 132.

Othmar G. Engelhardt, S. M. (2005). Association of the Influenza A Virus RNA-Dependent RNA Polymerase with Cellular RNA Polymerase II. . Journal of Virology 79(9): 5812–5818.

Palese P, S. M. (2001). Orthomyxoviridae: The Viruses and Their Replication. In H. P. Knipe D.M, 2001 Fields Virology, 5th edition. Lippencott Williams and Wilkins: Philadelphia .

Panzner, E. A., & Jansons, V. K. (1979). Control of in vitro cytotoxicity of positively charged liposomes. Journal of cancer research and clinical oncology, 95(1): 29-37.

Parnham, M. J., & Wetzig, H. (1993). Toxicity screening of liposomes.Chemistry and physics of lipids, 64(1-3): 263-274.

Patel, S. S., Patel, M. J., & Patel, N. M. (2010). Need, development and application of virosomal system in medicine. Int J Pharm Sci Nanotechnol,3: 1065-1074.

Paul E. Lund, 1. R.-S. (2010). Pseudovirions as Vehicles for the Delivery of siRNA. 27(3).

Perrin, P., Sureau, P., & Thibodeau, L. (1984). Structural and immunogenic characteristics of rabies immunosomes. Developments in biological standardization, 60: 483-491.

Persson, C. G., Erjefält, J. S., Korsgren, M., & Sundler, F. (1997). The mouse trap. Trends in pharmacological sciences, 18(12): 465-467.

Phillips, A. J. (2001). The challenge of gene therapy and DNA delivery.Journal of Pharmacy and Pharmacology, 53(9): 1169-1174.

Pöltl-Frank, F., Zurbriggen, R., Helg, A., Stuart, F., Robinson, J., Glück, R., & Pluschke, G. (1999). Use of reconstituted influenza virus virosomes as an immunopotentiating delivery system for a peptide-based vaccine. Clinical and experimental immunology, 117: 496-503.

Rambaut, A. P. (2008). The genomic and epidemiological dynamics of human influenza A virus. . Nature, 453(7195): 615-619.

Rambaut, A., Pybus, O. G., Nelson, M. I., Viboud, C., Taubenberger, J. K., & Holmes, E. C. (2008). The genomic and epidemiological dynamics of human influenza A virus. Nature, 453(7195): 615-619.

Reddy, A. T., Lakshmi, S. P., & Reddy, R. C. (2012). Murine model of allergen induced asthma. Journal of visualized experiments: JoVE, (63).

Ricciardolo, F. L., Nijkamp, F., Rose, V. D., & Folkerts, G. (2008). The guinea pig as an animal model for asthma. Current drug targets, 9(6): 452-465.

Ruigrok R. W. H, A. A. (1988). Studies on the Structure of the Influenza Virus Haemagglutinin at the pH of Membrane Fusion. . Journal of General Virology, 69: 2785-2795.

Saga, K., & Kaneda, Y. (2013). Virosome presents multimodel cancer therapy without viral replication. BioMed research international, 2013.

Sarachai, C., Sasipreeyajan, J., & Chansiripornchai, N. (2014). Characterization of avian influenza H5N1 virosome. Pak. Vet. J, 34: 201-204..

Saravanan. R, S. K. (n.d.). Virosomes : As a Versatile Carrier for Delivery System. 3(6). Scheule R K, .. (1986). Novel Preparation of Functional Sindbis Virosomes. . Biochemistry 25: 4223-4232.

Sarkar, D. P., Ramani, K., & Tyagi, S. K. (2002). Targeted Gene Delivery by Virosomes*. Liposome Methods and Protocols, 163-173.

Sarpong, S. B., Zhang, L. Y., & Kleeberger, S. R. (2004). A novel mouse model of experimental asthma. International archives of allergy and immunology, 132(4): 346-354.

Sato, K., Tanabe, T., & Ohya, M. (2010). How to classify influenza a viruses and understand their severity. Open Systems & Information Dynamics,17(03): 297-310.

Scheule, R. K. (1985). Novel Preparation of Functional Sindbis Virosomes. Schoen P1, C. A. (1999). Gene transfer mediated by fusion protein hemagglutinin reconstituted in cationic lipid vesicles. 6(5).

Seitzer, U., Bussler, H., Kullmann, B., Petersen, A., Becker, W. M., & Ahmed, J. (2005). Mouse strain specificity of the IgE response to the major allergens of Phleum pratense. International archives of allergy and immunology, 136(4): 347-355.

Sharma, R., & Yasir, M. (2010). Virosomes: a novel carrier for drug delivery.Int J Pharm Tech Res, 2(4): 2327-39.

Sharma, R., & Yasir, M. (2010). Virosomes: a novel carrier for drug delivery.Int J Pharm Tech Res, 2(4): 2327-39.

Shazly, G., Nawroth, T., & Langguth, P. (2008). Comparison of dialysis and dispersion methods for in vitro release determination of drugs from multilamellar liposomes. Dissolution technologies, 15(2): 7.

Shi, Y., Tan, Y., Mao, S., & Gu, W. (2014). Naringenin inhibits allergen-induced airway remodeling in a murine model of asthma. Molecular medicine reports, 9(4): 1204-1208.

Shinagawa, K., & Kojima, M. (2003). Mouse model of airway remodeling: strain differences. American journal of respiratory and critical care medicine,168(8): 959-967.

Siegle, J. S., Hansbro, N., Herbert, C., Yang, M., Foster, P. S., & Kumar, R. K. (2006). Airway hyperreactivity in exacerbation of chronic asthma is independent of eosinophilic inflammation. American journal of respiratory cell and molecular biology, 35(5): 565-570.

Skehel, J. J. (2000). Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annual review of biochemistry, 69(1): 531-569.

Stegmann, T., Morselt, H. W., Booy, F. P., Van Breemen, J. F., Scherphof, G., & Wilschut, J. (1987). Functional reconstitution of influenza virus envelopes. The EMBO journal, 6(9): 2651.

Steven J. Gamblin, J. J. (2010). Influenza Hemagglutinin and Neuraminidase Membrane Glycoproteins. Journal of Biological Chemistry, 285(37): 28403–28409.

Swirski, F. K., Sajic, D., Robbins, C. S., Gajewska, B. U., Jordana, M., & Stämpfli, M. R. (2002). Chronic exposure to innocuous antigen in sensitized mice leads to suppressed airway eosinophilia that is reversed by granulocyte macrophage colony-stimulating factor. The Journal of Immunology, 169(7): 3499-3506.

Takeda, K., Hamelmann, E., Joetham, A., Shultz, L. D., Larsen, G. L., Irvin, C. G., & Gelfand, E. W. (1997). Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell–deficient mice.The Journal of experimental medicine, 186(3): 449-454.

Tanaka, M., Shimbo, T., Kikuchi, Y., Matsuda, M., & Kaneda, Y. (2010). Sterile alpha motif containing domain 9 is involved in death signaling of malignant glioma treated with inactivated Sendai virus particle (HVJ-E) or type I interferon. International Journal of Cancer, 126(8): 1982-1991.

Temelkovski, J., Hogan, S. P., Shepherd, D. P., Foster, P. S., & Kumar, R. K. (1998). An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. Thorax, 53(10): 849-856.

Temelkovski, J., Hogan, S. P., Shepherd, D. P., Foster, P. S., & Kumar, R. K. (1998). An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. Thorax, 53(10): 849-856.

Timothy K. W, C. L. (2007). Biology of Influenza A Virus. Annual of the New York Acedmy of Sciences, 1102 (1): 1–25.

Tiwari, G., Tiwari, R., Sriwastawa, B., Bhati, L., Pandey, S., Pandey, P., & Bannerjee, S. K. (2012). Drug delivery systems: An updated review. International journal of pharmaceutical investigation, 2(1): 2.

Tiwari, G., Tiwari, R., Sriwastawa, B., Bhati, L., Pandey, S., Pandey, P., & Bannerjee, S. K. (2012). Drug delivery systems: An updated review.International journal of pharmaceutical investigation, 2(1): 2.

Tomley, F. M., & Cooper, R. J. (1984). The DNA Polymerase Activity of Vaccinia Virus 'Virosomes': Solubilization and Properties. Journal of general virology, 65(4): 825-829.

Toogood, J. H. (1987). Beta-blocker therapy and the risk of anaphylaxis.CMAJ: Canadian Medical Association Journal, 136(9): 929.

Tumpey, T. M., Maines, T. R., Van Hoeven, N., Glaser, L., Solórzano, A., Pappas, C., ... & García-Sastre, A. (2007). A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science,315(5812): 655-659.

Ueba, O. (1978). Respiratory syncytial virus. I. Concentration and purification of the infectious virus. Acta Med Okayama, 32(4): 265-272.

Vreede T F, F. E. (2010). The role of the influenza virus RNA polymerase in host shut-off. . Virulence, 1(5): 436–439.

Waelti, E., Wegmann, N., Schwaninger, R., Wetterwald, A., Wingenfeld, C., Rothen-Rutishauser, B., & Gimmi, C. D. (2002). Targeting her-2/neu with antirat Neu virosomes for cancer therapy. Cancer research, 62(2): 437-444.

Wagers, S. S., Norton, R. J., Rinaldi, L. M., Bates, J. H., Sobel, B. E., & Irvin, C. G. (2004). Extravascular fibrin, plasminogen activator, plasminogen activator inhibitors, and airway hyperresponsiveness. The Journal of clinical investigation, 114(1): 104-111.

Wagers, S., Lundblad, L. K., Ekman, M., Irvin, C. G., & Bates, J. H. (2004). The allergic mouse model of asthma: normal smooth muscle in an abnormal lung?. Journal of applied physiology, 96(6): 2019-2027.

Walczak, M., de Mare, A., Riezebos-Brilman, A., Regts, J., Hoogeboom, B. N., Visser, J. T., ... & Wilschut, J. (2010). Heterologous prime-boost immunizations with a virosomal and an alphavirus replicon vaccine.Molecular pharmaceutics, 8(1): 65-77.

Wan H, P. D. (2006). Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. . Virology, 346(2): 278-286.

Wan, H., & Perez, D. R. (2006). Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. Virology, 346(2): 278-286.

Wang, J., Li, F., Yang, M., Wu, J., Zhao, J., Gong, W., ... & Dong, L. (2014). FIZZ1 promotes airway remodeling through the PI3K/Akt signaling pathway in asthma. Experimental and therapeutic medicine, 7(5): 1265-1270.

Warner, R. L., Lukacs, N. W., Shapiro, S. D., Bhagarvathula, N., Nerusu, K. C., Varani, J., & Johnson, K. J. (2004). Role of metalloelastase in a model of allergic lung responses induced by cockroach allergen. The American journal of pathology, 165(6): 1921-1930.

Weckmann, M., Collison, A., Simpson, J. L., Kopp, M. V., Wark, P. A., Smyth, M. J., ... & Gibson, P. G. (2007). Critical link between TRAIL and CCL20 for the activation of TH2 cells and the expression of allergic airway disease. Nature medicine, 13(11):1308-1315.

Wegmann, M. (2008). Animal models of chronic experimental asthma—strategies for the identification of new therapeutic targets. Journal of Occupational Medicine and Toxicology, 3(1): 1.

Wenzel, S., & Holgate, S. T. (2006). The mouse trap: it still yields few answers in asthma. American journal of respiratory and critical care medicine, 174(11): 1173-1176.

Wiltschke, C., Wiedermann, U., Zurbriggen, R., Elandt, K., Bramswig, K., Jasinska, J., ... & Zielinski, C. C. (2008, May). A phase I study to evaluate safety, immunogenicity and antitumor activity of a HER2 multi-peptide virosome vaccine in patients with metastatic breast cancer. In ASCO Annual Meeting Proceedings (Vol. 26, No. 15_suppl, p. 3055).

Xueyong Zhua, R. M. (n.d.). Influenza Virus Neuraminidases with Reduced Enzymatic Activity That Avidly Bind Sialic Acid Receptors. 28.

Zhu, X., McBride, R., Nycholat, C. M., Yu, W., Paulson, J. C., & Wilson, I. A. (2012). Influenza virus neuraminidases with reduced enzymatic activity that avidly bind sialic acid receptors. Journal of virology, 86(24): 13371-13383.

Zosky, G. R., & Sly, P. D. (2007). Animal models of asthma. Clinical & Experimental Allergy, 37(7): 973-988.