EXPANSION OF VERO CELLS ON LOW-COST ULTRAVIOLET/OZONE (UVO) TREATED POLYSTYRENE (PS) MICROCARRIERS FOR NEWCASTLE DISEASE VIRUS(NDV) PRODUCTION

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ABSTRACT

EXPANSION OF VERO CELLS ON LOW-COST ULTRAVIOLET/OZONE (UVO) TREATED POLYSTYRENE (PS) MICROCARRIERS FOR NEWCASTLE DISEASE VIRUS(NDV) PRODUCTION

(Keywords: Newcastle disease, cell culture, halal, microcarriers)

Newcastle disease (ND) caused by Newcastle disease virus (NDV) is regarded as one of the most important poultry diseases in the world. ND vaccines until now is mainly produced by growing NDV vaccine strains in embryonated chicken eggs. This method poses many drawbacks, such as poor-quality control, high labor-intensity, time-consuming and requires big area for egg incubation. One method that has high possibility to overcome all the problems mentioned is by producing ND vaccines using animal cell culture. Animal cell culture offers many advantages over the traditional chicken eggs method. The method is rapid, convenient, less expensive than eggs, supports easy scale up and it allows evidence of viral proliferation to be examined microscopically. Further, with the advent of microcarrier cell culture technology, high density cell culture is achievable and virus yield produced from the cell culture is expected to be more than the embryonated chicken eggs method or at least on par.

The present work aims to prepare a model to mass produce mesogenic La Sota strain of NDV using self-prepared ultraviolet/ozone (UVO) treated polystyrene (PS) microcarriers in spinner vessel culture. First, Vero cell culture were sequentially adapted from using Dulbecco's Modification of Eagle Medium (DMEM) to Virus-Production Serum Free Medium (VP-SFM). Adapted Vero cells were later used as hosts to propagate La Sota NDV in T-flask cultures. Concurrently, PS microspheres were produced using oil in water (O/W) emulsification-solvent evaporation method, and then treated with UVO to introduce functional groups that can promote cell adherence and growth. UVO treated PS microcarriers were characterized by toluidine blue O (TBO) assay, Fourier-transformed infra-red (FTIR) and scanning electron microscopy (SEM).

From experiments, it was revealed that, adaptation from 100% DMEM to culture in 100% VP-SFM has resulted higher maximum cell concentration, from 1.063×10^6 cells/ml to 1.595×10^6 cells/ml, respectively. However, La Sota propagation in Vero cells that were cultured in 100% VP-SFM yielded 0 HA titer for 5 consecutive adaptations. On the other hand, analysis of UVO treated by PS microcarriers by TBO assay and FTIR showed increased surface oxygen concentration after UVO treatment. Results from spinner flask culture also revealed that UVO treated PS microspheres support the growth of Vero cells to high cell density.

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TABLE OF CONTENTS

Acknowledgements i
Abstract ii
List of Tables iv
List of Figures v
List of Abbreviations vi

CHAPTER ONE: INTRODUCTION 1

CHAPTER TWO: LITERATURE REVIEW 6

CHAPTER THREE: MATERIALS AND METHODS 44

CHAPTER FOUR: RESULTS AND DISCUSSION 59

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS . 72

REFERENCES 73

APPENDIX: LIST OF PUBLICATIONS 79

LIST OF TABLES

Table No.		Page No.
4.1	The maximum cell concentration (cells/mL) and doubling time (hr) of cells at different concentration of medium.	62
4.2	EDX data showing carbon and oxygen content on untreated and UVO treated PS microspheres	67

LIST OF FIGURES

Figure No.		Page No.
2.1	Schematic diagram of Newcastle disease virus particle.	13
2.2	Electron micrograph of negatively stained pleomorphic Newcastle disease virus (strain Beaudette C) particles obtained from supernatant of infected chicken embryo fibroblast cells.	14
2.3	Genetic map of genomic RNA of NDV.	16
2.4	Schematic diagram of Paramyxovirus transcription and replication.	23
2.5	The life cycle of NDV.	25
2.6	Phases of growth in cell culture.	41
3.1	An overview of experimental studies.	67
3.2	Assembly of the ultraviolet/ozone (UVO) system	
3.3	Counting grid of haemacytometer.	75
4.1	Detached cells at 10X magnification.	60
4.2	Cells at 12th hour under 20X magnification	60
4.3	Cells at 120th hour under 20X magnification.	60
4.4	The graph of concentration of cells (cells/mL) against time (hour).	62
4.5	ATR/IR spectra of untreated and UVO treated PS microspheres	65
4.6	Subtraction result of ATR/IR plot; UVO treated PS minus untreated PS microspheres	65
4.7	SEM photographs of untreated PS microspheres at different	67
4.8	SEM photographs of UVO treated PS microspheres at different magnifications	68
4.9	Growth profile of Vero cells grown on untreated PS and UVO treated PS microcarriers	69

4.10 Vero cell attachment on UVO treated PS microcarriers viewed under inverted light microscope at different times of culture: a) 2 hours and b) 60 hours 70

4.11 Vero cell attachment on untreated PS microcarriers viewed under inverted light microscope at different times of culture: a) 0 hours and b) 60 hours 70

4.12 SEM photographs of Vero cell attachment on UVO treated PS microcarriers at different magnifications 71

LIST OF ABBREVIATIONS

APMV-1	Avian Paramyxovirus Serotype 1
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
$CHCl_3$	Chloroform
CHO	Chinese Hamster Ovary
CO	Carbonyl
CO2	Carbon dioxide
COOH	Carboxyl/carboxylic acid
CPE	Cytopathic Effect
DEAE	Diethylaminoethyl
DMEM	Dulbecco's Modification of Eagle's Medium
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
FLFAM	Federation of Livestock Farmer's Associations of Malaysia
FTIR	Fourier Transform Infrared Spectroscopy
HA	Haemagglutination Assay
HN	Haemagglutinin/Neuraminidase Protein
IFN	Interferon
M	Matrix Protein
MOI	Multiplicity of Infection
MVP	Malaysia Vaccine Pharmaceuticals Sdn. Bhd.
ND	Newcastle Disease
NDV	Newcastle Disease Virus
OIE	Office de Internationale Epizooties
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
PFU	Plaque Forming Unit
PS	Polystyrene
PTFE	Polytetrafluoroethylene
RBC	Red Blood Cells
SEM	Scanning Electron Microscope
SPF	Specific Pathogen Free
TBO	Toluidine Blue O
$TCID_{50}$	50% Tissue Culture Infective Dose
UPM	Universiti Putra Malaysia
UV	Ultraviolet
UVO	Ultraviolet/ozone
VP-SFM	Virus Production Serum Free Medium
VRI	Veterinary Research Institute

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Newcastle disease (ND) caused by Newcastle disease virus (NDV) is regarded as one of the most important viral diseases of poultry in the world (Spradbrow, 1987; Adene, 1990). The disease has a worldwide distribution, and is a major threat to the poultry industries due to the huge economic loss associated with it. ND which had its first outbreak in 1926, in Java, Indonesia (Kraneveld, 1926), was named after a place in England where it was rediscovered a year later (Doyle, 1927). About 27 of the 50 orders of birds have been reported to be susceptible to natural or experimental infections of NDV (Yusoff and Tan, 2001). Chickens are the most susceptible host, in which the severity of the disease may vary from mild infection with no apparent clinical signs to a severe form causing 100% mortality (Huang, et al., 2003).

NDV which is also designated as avian paramyxovirus 1 (APMV-1) is a member of the genus Avulavirus of the family Paramyxoviridae, in the order Mononegavirales (Mayo, 2002). Other important pathogens included in this family are the mumps virus, human parainfluenza virus, sendai virus, simian virus 5 and recently emerging nipah and hendra viruses. Electron microscopic examinations reveal that virus particles of members of this group have pleomorphic structure (Yusoff and Tan, 2001). They generally appear as rounded particles with diameters around 100-500 nm but often filamentous forms of about 100 nm across and variable length are seen (Alexander, 1988b).

The virus is enveloped and contains a single linear strand, non-segmented negative sense RNA molecule of 15,186 nucleotides (nt) as its genome (Krishnamurthy and Samal, 1998; de Leeuw and Peeters, 1999). The genomic RNA of the virus is consists of six genes, which encode for at least eight proteins (Peeples, 1988). The genes are arranged in tandem in the order of 3' - NP-P-M-F-HN-L- 5' which encode for nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and large polymerase protein (L), respectively (de Leeuw and Peeters, 1999).

Based on the clinical signs and severity of disease produced in chickens, strains of NDV are classified into three major pathotypes; lentogenic, mesogenic, and velogenic. Lentogenic strains cause mild or avirulent infections that are largely limited to the respiratory. Strains of intermediate virulence that cause respiratory disease with moderate mortality are termed mesogenic while strains that are highly virulent causing 100% mortality in chickens are termed as velogenic. Velogenic strains can be further categorized into two types: viscerotropic and neurotropic. Viscerotropic velogenic strains produce lethal hemorrhagic lesions in the digestive tract whereas neurotropic velogenic strains produce neurological and respiratory disorders (Spradbrow, 1987; Alexander, 1997).

NDV is usually transmitted through 'traditional routes' such as direct animal contact, contaminated feed, water, implements, transport contact or transmission by people (Alexander, 1988a). Airborne transmission is also considered as one of the important routes for spreading of ND. ND outbreaks in England in 1970–1971 (Hugh-Jones et al., 1973) and epidemic in Northern Ireland in 1973 (McFerran, 1989) was believed to be caused by airborne NDV. The success of NDV transmission through this route depends on many environmental factors, such as temperature, humidity, and stocking density. Delay et al. (1948) and Hietala et al. (2005) have

demonstrated that NDV can be recovered from poultry environment air samples. Although airborne NDV is a major threat to poultry farms, they are usually heat labile and readily destroyed by exposure to high temperature (>50°C) and also by ultraviolet rays in sunlight (Lomniczi, 1975).

1.2 PROBLEM STATEMENT AND ITS SIGNIFICANCE

ND has produced huge economic losses to many economies worldwide. Before the emergence of the highly pathogenic Asian H5N1 influenza virus, the economic impact of ND has remained unsurpassed by other poultry viruses. In developed countries where the poultry industries are well established, not only outbreaks of ND are extremely costly but preventive measures taken to avoid the disease also represent continuing losses to the industry (Alexander and Senne, 2008). In 2002, an outbreak caused by a virulent strain of NDV in California, USA has caused $200 million worth of losses from the depopulation of birds (Kapczynski and King, 2005). Furthermore countries with ND free status also have to bear huge costs from repeated testing to maintain the status and for purposes of trade. While in many developing countries, recurring outbreaks has made ND a major limiting factor to the growth of commercial poultry industries and establishment of trade links (Alexander and Senne, 2008). Yusoff (2008) has reported that in Malaysia alone, the losses caused by ND amount to over RM 100 million in costs annually.

Until now, there is no treatment or method to eradicate ND. Prevention is by importing birds from disease free flocks or through vaccination that must continue throughout the life of the bird (Alexander, 1992; OIE, 2002). Traditionally, ND vaccines have been produced by growing vaccine virus strains in embryonated chicken eggs. NDV is harvested from the allantoic fluid and processed to create a vaccine (Gallili and Nathan, 1998). This traditional method however poses some drawbacks, such as poor quality control, high labor-intensity, time consuming, needs high

amount of specific pathogen-free eggs, and requires big area for the incubation of eggs. Besides the process is slow and difficult to scale-up, so large strategic stocks must be kept to respond in cases of epidemics (Souza, et al., 2009).

In Malaysia there are only two agencies that produce vaccines for ND; the Veterinary Research Institute (VRI) and also the Malaysia Vaccine Pharmaceuticals (MVP) Sdn. Bhd. Both agencies use the cumbersome embryonated chicken eggs method which has resulted to insufficient amount and types of vaccines required to provide immunities to almost 516 million birds (broilers) produced in Malaysia in 2009 (FLFAM, 2010). To fulfill high local demand of ND vaccines, Malaysia has to import vaccines from overseas and this step poses a great risk because newer strains may be introduced to the local poultry industries.

1.3 RESEARCH HYPOTHESES

One method that has high possibility to overcome all the problems mentioned is by producing ND vaccines by using animal cell culture. Animal cell culture offers many advantages over the traditional chicken eggs method. The method is rapid, convenient, less expensive than eggs, supports easy scale up and also it allows evidence of viral proliferation to be examined microscopically. Many cell substrate systems have been reported to be able to support the growth of NDV (de Leeuw and Peeters, 1999; DiNapoli, et al., 2007; Ravindraa, et al., 2008). In addition, more mechanized cell culture methodology allows virus particles to be produced with the highest control and quality. There are also numerous types of culture medium and bioreactor systems that are easily modified to mimic the environment of virus replication in eggs. And finally with the advent of microcarrier cell culture technology, high density cell culture is achievable and virus

yield produced from the cell culture is expected to be more than the embryonated chicken eggs method or at least on par.

1.4 RESEARCH OBJECTIVES

The general objective of this study is to develop a model for producing Newcastle disease virus (La Sota strain) for preparation of live vaccine by using cell culture as an alternative to the traditional embryonated chicken eggs method. The specific objectives based on the reasons discussed are as follows:

i. To adapt Vero host cells in Virus-Production Serum Free Medium (VP-SFM).

ii. To produce and characterize UVO treated PS microcarriers.

iii. To mass produce ND virus in spinner vessel using UVO treated PS microcarriers.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

The first outbreaks of the severe disease of poultry known as Newcastle disease (ND) occurred in 1926, in Java, Indonesia (Kraneveld, 1926), and in Newcastle-upon-Tyne, England (Doyle, 1927). The links between these initial outbreaks has been postulated by many authors. Many has considered that the presence of the disease in England was resulted from transportation to the port of Newcastle upon Tyne from South East Asia by ship, either in frozen meat or as a result of the practice of keeping live chickens on board for eggs and meat (Alexander, 1988a). There are also reports of the disease outbreaks earlier than 1926. Macpherson (1956) considered the death of all the chickens in the Western Isles of Scotland in 1896 to be attributable to ND. The disease also appears to have been present in Korea in 1926 although Levine (1964) citing Ochi and Hashimoto (1929) indicated that the disease may have been present in Korea as early as 1924. ND outbreak also occured in Ranikhet in India in July, 1927, but the links with shipping could be excluded as the the town is situated in the foothills of Himalaya mountains 600 miles from the sea (Edwards, 1928).

The name Newcastle disease was given by Doyle to a highly pathogenic disease seen in chickens in a farm near Newcastle upon Tyne and hence the name. Doyle considered the name as temporary because he wanted to avoid a descriptive name that might be confused with other diseases (Doyle, 1935). Nevertheless no better name has evolved over the past 84 years although the synonym avian paramyxovirus type 1 (APMV-1) has gained some popularity in recent years. Nowadays APMV-1 is often used to refer the low virulent strains while Newcastle disease is

reserved for virulent strains as based on definitions used by the World Health Organisation and other international agencies (Alexander, 1988b).

Several years after the 1926 outbreak, many less severe diseases emerged, caused by viruses indifferentiable from Newcastle disease virus by conventional methods. In the United States a relatively mild respiratory disease frequently with nervous signs was first described in 1930s (Beach, 1942). The virus which is termed as pneumoencephalitis is indistinguishtable from NDV by serological tests (Beach, 1944). Since then, numerous NDV isolations that produced an extremely mild disease or no evidence of disease in chickens have been made around the world (Asplin, 1952).

2.1.1 Host Range

It has been reported that over 250 species from 27 of the 50 orders of birds are susceptible to natural or experimental infection of NDV. It is highly probable that all species of birds are susceptible to NDV infection but the outcome of the disease varies differently in different species. Ducks for example tend to display few signs of the disease even when infected with strains that are very virulent to chickens (Kaleta and Baladauf, 1988).

2.1.2 Pathotypes

Newcastle disease is complicated in that different strains and isolates of the virus may cause quite distinct signs and enormous severity of disease even in the same host species. Based on the clinical signs in chicken, Beard and Hanson (1984) have divided NDV into five pathotypes:

i. Doyle's form (Doyle, 1927), an acute, lethal infection of all ages of chickens. Clinical manifestation occur predominantly in the intestinal tract leading to a severe enteritis

mainly characterised by diarrhoea which is often green in colour and this form of disease has been termed as viscerotropic velogenic Newcastle disease virus (VVND).

ii. Beach's form (Beach, 1942), an acute, often lethal infection of chicken on all ages. High mortality following respiratory distress and central nervous system disorders hence termed as neurotropic velogenic Newcastle disease virus (NVND).

iii. Beaudette's form (Beaudette and Black, 1946) that appears to be a less pathogenic form of NVND in which deaths usually are only seen in young birds. The main clinical signs of NDV infection of this form are drop in egg production, poor egg quality and decreased feed consumption. Virus causing this type of infection is termed as mesogenic and may be used as secondary live vaccine.

iv. Hitchner's form (Hitchner and Johnson, 1948) is represented by mild or inapparent respiratory infections caused by viruses of the lentogenic pathotypes. Usually apathogenic in adult birds and commonly used as live vaccines.

v. Asymptomatic-enteric form (Alexander, 2008) , which is mainly a gut infection with lentogenic viruses causing inapparent enteric infection. Some live commercial vaccines are of this pathotype.

2.1.3 Transmission

Newcastle disease infection can take place by virus inhalation of aerosols, ingestion of contaminated faeces (slow) or contact with mucous membranes, especially the conjunctiva. The mode of transmission of the disease from one bird to another therefore depends on the organs in which the virus multiplies and this may vary with viral pathotype. Birds displaying respiratory disease presumably shed virus in aerosols of mucus that may be inhaled by or contact susceptible

birds. Viruses that are usually restricted to intestinal replication may be transferred by ingestion of contaminated faeces, either directly or in contaminated food or water, or by the production of small infective particles produced from dried faeces that may be inhaled or impinge on mucous membranes (Alexander, 2009). In a community of closely situated birds, virus that is transmitted through the respiratory route may spread with alarming rapidity. While virus that is transmitted by the oral/faecal route may spread slowly especially when birds are not in direct contact (Alexander, 1988a).

2.2 NEWCASTLE DISEASE VIRUS

2.2.1 Classification

The virus order Mononegavirales (i.e. the single-stranded, nonsegmented, negative-sense RNA viruses showing helical capsid symmetry) is consists of Paramyxoviridae, Filoviridae and Rhabdoviridae virus families. The family Paramyxoviridae is separated into two subfamilies Pneumovirinae and Paramyxovirinae (Lamb et al. 2005). The subfamily pneumovirinae has two genera: Pneumovirus and Metapneumovirus (Alexander and Senne, 2008) while the subfamily Paramyxovirinae has five genera: Rubulavirus, which includes the mumps virus, mammalian para-influenzas 2 and 4; Respirovirus containing Sendai virus, mammalian para-influenza viruses 1 and 3; Morbillivirus, which includes measles, distemper and rinderpest; Henipavirus, formed from the Nipah and Hendra viruses; and the Avulavirus genus, formed from Newcastle disease virus (NDV), and other avian paramyxoviruses (Lamb and Kolakofsky, 1996).

2.2.2 Virion composition

The molecular weight of Newcastle disease virus is about 500 x 10^6. It has density of 1.18-1.20 g/ml in sucrose and 1.18-1.31 g/ml in caesium chloride. Its negative sense single stranded nonsegmented RNA has molecular weight of 5 x 10^6 which makes up about 0.5% by weight of the virus particle. Lipids which is found in the lipid bilayer membrane constitutes 20-25% of the virus by weight. The lipids are derived from plasma membrane of the host cell and has similar composition to that of host cell membranes. Carbohydrates constitutes 6% of virus dry weight and they present in the glycoproteins of NDV (Alexander, 1997).

Nucleotide sequencing of the NDV genome has shown it to consists of 15,186 nucleotides (Krishnamurthy and Samal, 1998) which contains six major genes that encode six structural proteins; large protein (L), haemagglutinin-neuraminidase protein (HN), fusion protein (F), matrix protein (M), phosphoprotein (P) and nucleocapsid protein (NP) as well as two non-structural proteins, the W and V proteins (Lamb and Kolakofsky, 1996). Figure 2.1 shows a schematic diagram of the NDV.

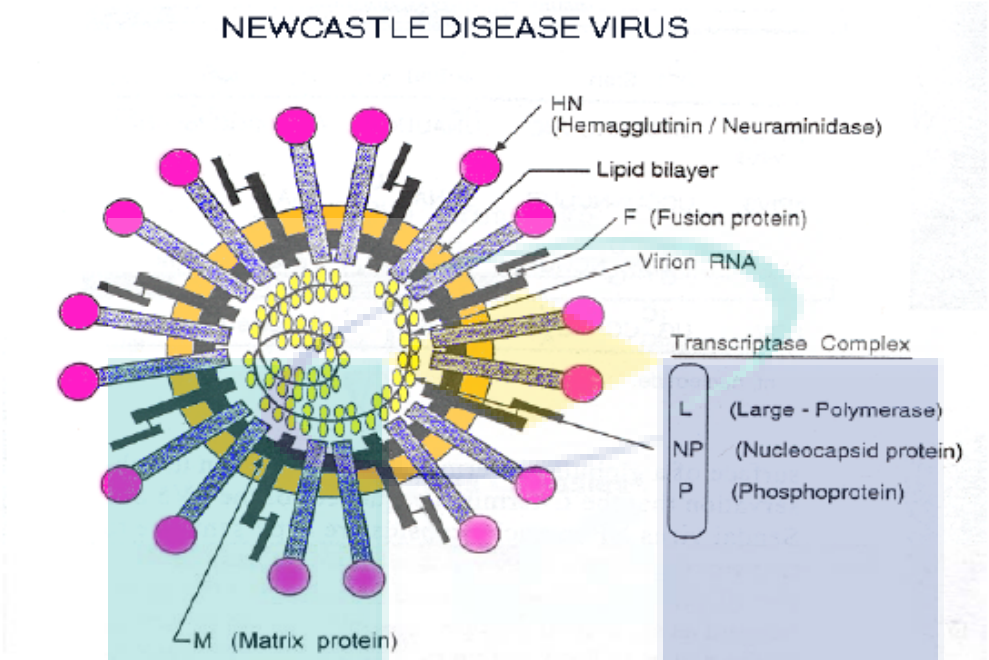

Figure 2.1: Schematic diagram of Newcastle disease virus particle (not drawn to scale). (Figure from dissertation by Panda, A. 2003. Role of Hemagglutinin-Neuraminidase Protein in Newcastle Disease Virus Pathogenesis (Doctoral dissertation). Retrieved from http://drum.lib.umd.edu/bitstream/1903/123/1/dissertation.pdf

2.2.3 Morphology

Electron microscope examinations of Newcastle disease virus reveals very pleomorphic virus particles. Generally, they are spherical with diameters around 100-500 nm. Occasionally, filamentous particles of approximately 100 nm in diameter and variable length can be seen (Yusoff and Tan, 2001). The virion is enveloped with lipid bilayer membrane which mature naturally by budding through membrane of the host cell. The envelope has spike-like projections, 8 nm in length that comprise of two different glycoproteins, the haemagglutinin-neuraminidase (HN) and fusion (F) proteins that are embedded in the lipid bilayer membrane. The F and HN are the main targets of the immune response of NDV and either of these proteins can induce protective immunity (Stone-Hulslander and Morrison, 1997). Beneath the lipid bilayer membrane is a layer

of M protein which is relatively hydrophobic and non-glycosylated. This M protein is thought to play an important role in the assembly of the virus by interacting with the nucleocapsid protein (NP) of NDV, lipid bilayer membrane and also the HN and F proteins that are exposed on the inner structure of the membrane (Garcia-Sastre et al., 1989). Nucleocapsid protein (NP) of NDV has herringbone-like structure which can be seen as free or emerging from disrupted virus particles in most electron micrographs. This herringbone like structure comprises thousands of NP subunits and are tightly associated with several copies of phospoprotein (P) and large protein (L). Together these three RNA associated proteins and RNA genome which are located in the central hollow of the herringbone like nucleocapsid form the viral transcriptase complex that serves as the minimum infectious unit (Krishnamurthy and Samal, 1998; de Leeuw and Peeters, 1999). Figure 2.2 depicts an electron micrograph of the virus particle.

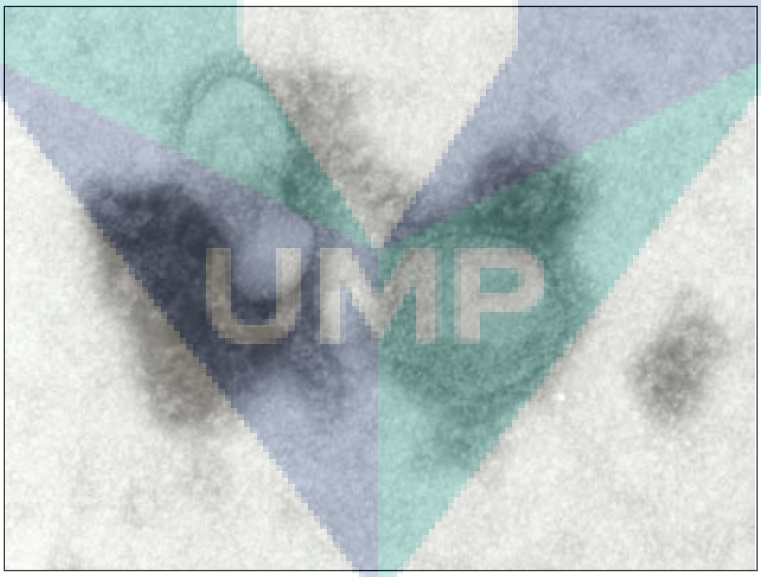

Figure 2.2: Electron micrograph of negatively stained pleomorphic Newcastle disease virus (strain Beaudette C) particles obtained from supernatant of infected chicken embryo fibroblast cells. (Figure from dissertation by Rout, S.N. 2007. The role of Newcastle disease virus internal proteins in pathogenesis (Doctoral dissertation). Retrieved from Dissertations and Theses database. (UMI No. 3277526)).

2.2.4 Genome organization

The NDV genome consists of six major genes that encode the structural proteins in the order 3'-NP-P-M-F-HN-L-5' as well as two non-structural proteins, the W and V proteins (Peeples, 1991; Steward et al., 1993). These non-structural proteins are resulted from transcriptional modification of the P gene mRNA, by insertion of one or two G residues to result in frameshifts. The genomic RNA contains a 3' extracistronic region of 55 nucleotides, known as the leader sequence, and a 5' extracistronic region of 144 nucleotides, known as the trailer sequence. These sequences are essential in the regulation of NDV replication, transcription, and encapsidation of the genomic and antigenomic RNAs (Lamb and Kolakofsky, 1996). At the start and end of each gene are conserved transcriptional control sequences, known as the gene start and gene end sequences, respectively. The six structural genes are separated by intergenic regions which vary in length from 1-47 nucleotides. These intergenic regions probably involved in terminating mRNA transcription from the preceding gene, before initiating transcription of the subsequent gene (Chambers et al., 1986; Krishnamurthy and Samal, 1998) (Figure 2.3).

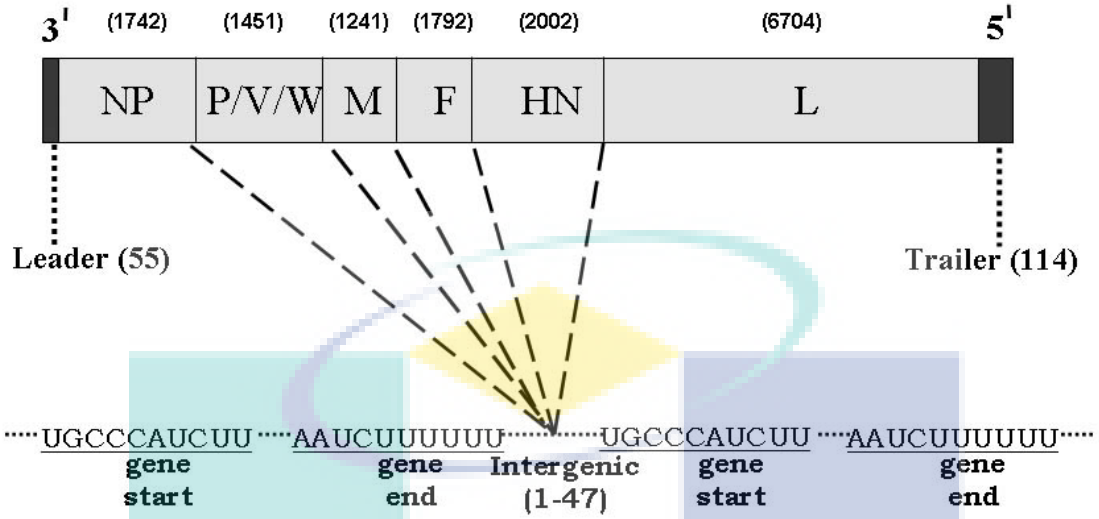

Figure 2.3: Genetic map of genomic RNA of NDV. (Figure from dissertation by Rout, S.N. 2007. The role of Newcastle disease virus internal proteins in pathogenesis (Doctoral dissertation). Retrieved from Dissertations and Theses database. (UMI No. 3277526)).

2.2.5 Nucleocapsid and its associated protein

The nucleocapsid protein (NP) which is tightly bound to the viral genomic RNA form a core structure to which the phosphoprotein (P) and the large polymerase protein (L) are loosely bound. These three proteins form the transcriptive-replicative complex which are the active polymerase complex and minimum infectious unit of NDV (Lamb and Kolakofsky, 1996).

2.2.5.1 Nucleocapsid Protein (NP)

The NP protein is the most abundant protein in the virus. This protein which has 489 amino acid residues is encoded by the NP gene which comprises a single open reading frame (ORF) of 1742 nucleotides. The molecular weight of NP protein is predicted to be 54 kilodaltons (kD) (Krishnamurthy and Samal, 1998). The NP protein serves several functions in viral replication including encapsidation of viral genomic RNA thus making the nucleocapsid RNAase resistant,

association with P and L protein during transcription and replication and interaction with M protein during virus assembly. The intracellular concentration of unassembled NP proteins plays major role in the switching of transcription to replication of the viral genome (Blumberg et al., 1981).

2.2.5.2 Phosphoprotein (P)

The P protein of NDV is the most heavily phosphorylated viral protein. It is highly acidic in nature and is produced by unedited version of P gene ORF (McGinnes et al., 1988; Steward et al., 1993). The protein which comprises of 395 amino acid residues and having molecular weight of 53 kD is encoded by 1451 nucleotides long P gene. The co-transcriptional insertion of one G nucleotide to the editing site of the P gene ORF produces an mRNA which encodes the V protein, whereas addition of two G nucleotides produces an mRNA that encodes the W protein (Lamb and Kolakofsky, 1996). Nucleotide sequence analysis of NDV P gene shows that the protein is rich in serine and threonine residues thus acting as potential phosphorylation sites. The P protein associates with NP and L proteins forming viral polymerase complex which functions as a transcriptive and replicative factor. It also associates with unassembled NP (NP^0) to form P-NP^0 complexes thus preventing nonspecific assembly or self aggregation of NP^0 proteins (Steward et al., 1993).

2.2.5.3 Large Protein (L)

The L protein is the largest structural protein with least abundance in the virion core (about 50 copies per virion) and is a major component of the RNA-dependent RNA polymerase in negative strand RNA viruses (Banerjee, 1987; Tordo et al., 1988). The L gene is 6704 nucleotides long and its ORF of 6615 nucleotides encodes L protein which comprises of 2204 amino acid residues and

having molecular weight of 242 kD. Together P and L proteins form a complex, and this complex is required for polymerase activity with NP: RNA templates. The L protein is also responsible for 5' capping and 3' polyadenylation of the nascent viral mRNAs (Yusoff and Tan, 2001).

2.2.6 Matrix Protein (M)

The M protein is the most plentiful protein inside the virion particle. The protein which comprises of 364 amino acid residues is encoded by 1241 nucleotides long M gene. The molecular weight of M protein is 40 kD (Chambers et al., 1986). The M protein interacts with the cytoplasmic tails of the the lipid bilayer, integral membrane proteins and the nucleocapsid, and is considered to be the central organizer of viral morphogenesis. The self-association of M proteins and its contact with the nucleocapsid may be the driving force in forming a budding virus particle (Peeples, 1991).

2.2.7 Envelope gylcoproteins

The envelope of NDV possesses two integral membrane glycoproteins namely haemagglutinin-neuraminidase (HN) glycoprotein that is responsible in the attachment of the virus to the host cell membrane and the fusion (F) glycoprotein that mediates pH-independent fusion of the viral envelope with the plasma membrane of the host cell.

2.2.7.1 Haemagglutinin-neuraminidase Protein (HN)

The HN glycoprotein of NDV is a multifunctional protein and a major antigenic determinant of the virus. The protein which has predicted molecular weight of 74kD is comprised of 577 amino acid residues and is encoded by 2002 nucleodtides long HN gene (Chambers et al., 1986). HN protein binds with sialic acid thus responsible for the adsorption of the virus to sialic acid

containing receptors. Furthermore HN mediates enzymatic cleavage of sialic acid (neuraminidase activity) from the surface of the virion as well as infected host cell membranes. Other than hemagglutinin and neuraminidase activities, it also has fusion promotion activity through interaction with the F glycoprotein of NDV (Lamb and Kolakofsky, 1996). The HN protein is a type II integral membrane protein with a single hydrophobic domain at N-terminal region which composed of cytoplasmic domain, followed by the transmembrane and the stalk region. The C-terminus end of the protein is comprised of the globular head or the ectodomain. This region of the HN protein is the main site of virus attachment to host cells (Deng et al., 1999; Crennell et al., 2000).

2.2.7.2 Fusion Protein (F)

The F glycoprotein of NDV is composed of 553 amino acid residues and is encoded by 1792 nucleotides long F gene. The protein mediates viral penetration by fusion between the viral envelope and the host cell plasma membrane, in a pH dependent manner. The fusion creates pores on plasma membrane through which the viral nucleocapsid is delivered into the host cell cytoplasm. After fusion, the nucleocapsid is delivered into the cytoplasm. Later in infection, the F protein expressed at the plasma membrane of infected cells can mediate fusion with neighboring cells to form giant multinucleated cell or syncytia. Syncytia formation is a distinctive feature of NDV infection in host cells. It is a typical cytopathic effect caused by the virus which can lead to tissue necrosis and might also be a mechanism of virus spread. The F protein is a type I integral membrane protein and is synthesized as an inactive precursor (F_0) that requires host-cell proteolytic enzyme(s) for its cleavage. This cleavage of F_0 yields two biologically active subprotein F_1 and F_2 which are connected to each other by disulfide link (Scheid and Choppin,

1974). F_0 has a predicted molecular weight of around 66 kD whereas F_1 and F_2 are approximately, 55 kD and 12.5 kD, respectively. The cleavage of F_0 is the key determinant for pathogenicity of paramyxoviruses. Viruses that have multiple basic amino acid residues at their cleavage site of F protein are cleaved by intracellular subtilisin-like proteases such as furin. However, viruses that have single basic amino acid residue at their cleavage site of F protein cannot be cleaved intracellularly and require exogenous proteases for cleavage activation (Scheid and Choppin, 1974; Ortmann et al., 1994).

2.2.8 Stages of replication of NDV

The replication strategies of NDV are very similar to that of other non segmented negative-strand RNA viruses of paramyxoviridae. Initiation of infection starts with virus attachment to the host cell receptors followed by subsequent fusion into host cellular membrane and entry of the viral nucleocapsid into the host cell cytoplasm. All aspects of replication of NDV occurs in the host cell cytoplasm. During the late stages of NDV infection, there will be a complete shutdown of host cell macromolecule synthesis. At the end of infection, progeny viruses mature by budding through the plasma membrane. Cleavage of the F_0 precursor glycoprotein to F_1 and F_2 by host cell proteases is required for progeny virus to become infective (Nagai et al., 1976; Garten et al., 1980).

2.2.8.1 Virus attachment, fusion, and entry

NDV infection to host cells begins with the attachment of HN protein to the specific cell receptors containing sialic acid. Neuraminidase activity of HN protein and the availability of sialic acid on most cell surface receptors allow NDV to access wide range of host cells. During adsorption of the virus, the envelope of virus fuses with the host cell plasma membrane at neutral pH. This fusion

is mediated by F protein in a pH independent manner. Upon fusion, the M proteins underneath the membrane become dissociated from the nucleocapsid through an unknown mechanism, releasing the viral nucleocapsid into the cytoplasm to begin replication and transcription (Huang et al., 1980).

2.2.8.2 Transcription

A naked negative-sense RNA genomes of paramyxovirus are non-infectious in uninfected host cells due to lack of RNA-dependent RNA polymerase (RDRP) activity. All viral mRNA transcription begins at 3' end of the genome. Once the nucleocapsid is released into the host cell cytoplasm, the RDRP complex enters at 3' end of viral genome promoter and short (+) strand leader RNA is synthesized first. The leader sequence has the regulatory elements needed for gene expression. After synthesis of the leader RNA, re-initiation of mRNA synthesis begins at NP gene start sequence. Majority of RDRP complexes terminates transcription at gene end (GE) sequence, but some of them bypass the GE signal to continue transcription of downstream genes. This sequential start and stop mechanism leads to a gradient of mRNA abundance that reduces according to the distance of the location of a particular gene from the 3′ end of the genome (Cattaneor et al., 1987). The mRNA produced are capped and polyadenylated in nature. The intergenic regions located in between the genes are not transcribed. The exact role of these intergenic regions is unknown (Weiss and Bratt, 1974).

2.2.8.3 Replication

Genome replication occurs by the synthesis of a full-length (+) antigenome, which in turn functions as a template for the production of (-) genome. After primary transcripts have been translated and

viral proteins have been accumulated, (+) antigenome synthesis begins. During this process, RDRP which was engaged in mRNA synthesis till now, copies the same genomic template, but this time, ignores all the functional signals such as start-stop signals and editing sites, and synthesizes an exact complimentary copy (Kolakofsky and Blumberg, 1982; Nagai, 1999). Together the (+) genome and (-) antigenome are assembled into encapsidated nucleocapsid. Several studies have reported that the leader and trailer regions of the genome contain specific sequences for encapsidation. The switching of transcription to replication and vice versa is tightly regulated. When unassembled NP is limiting, RDRP is preferentially engaged in mRNA synthesis, raising the intracellular levels of unassembled NP and all other viral proteins. When unassembled NP levels are sufficient, some RDRP switches from transcription to replication, thereby reducing the levels of unassembled NP, as each initiation of encapsidation uses many NP monomers to finish the assembled genome chain (Blumberg and Kolakofsky, 1981). The RNA synthesis of NDV is shown in Figure 2.4.

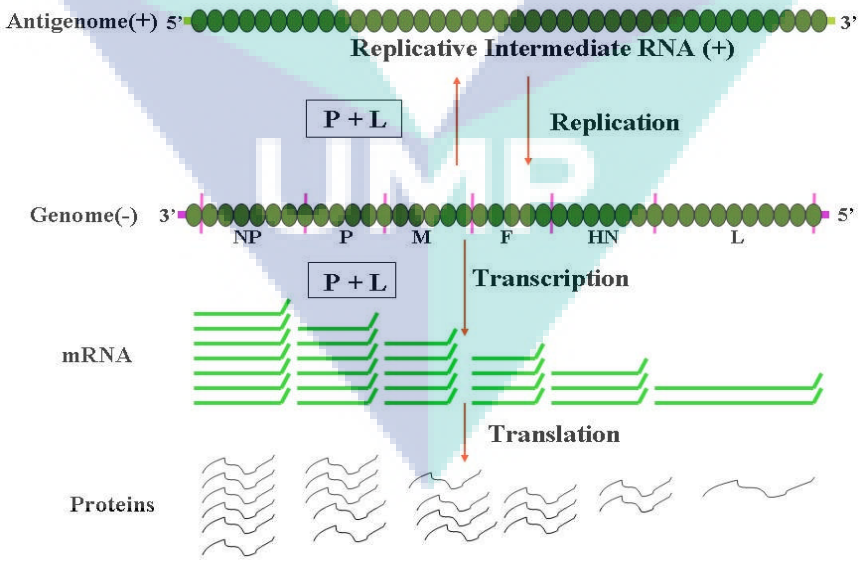

Figure 2.4: Schematic diagram of Paramyxovirus transcription and replication. (Figure modified from Collins *et al*., 1996. Respiratory Syncytial virus, In Fields, B.N. Knipe, D.M. and Howley, P.M. (ed). Virology 3rd ed. Raven Press, New York).

2.2.8.4 Virus assembly and release

The assembly of the nucleocapsid core takes place in the host cell cytoplasm. The nucleocapsids are believed to be assembled in two steps: first, free NP subunits are tightly encapsidated with viral genomic RNA to form helical ribonucleoprotein (RNP) structure, and then P and L proteins are loosely bound to RNP forming transcriptase complex (Kingsbury et al., 1978). The assembly of the viral envelope takes place at the cell surface. The membrane glycoproteins, HN and F_0, are synthesized in the rough endoplasmic reticulum, whereas the rest of the viral structural proteins (NP, P, L and M) and the non-structural proteins (V and W) are produced in the cytoplasm. The glycoproteins undergo a number of post-translational modifications, such as glycosylation and formation of a disulphide bond, when they are transported across the endoplasmic reticulum and Golgi apparatus. The cleavage of F_0 into two disulphide-linked fragments, F_1 and F_2, occurs in the Golgi apparatus. After successful maturation, finally the glycoproteins are transported to the surface of the cell membrane through vesicles where the assembly of the envelope takes place and subsequently viruses are released through budding (Feller et al., 1969; Doms et al., 1993). The detailed mechanism of NDV assembly and its release at the cell membrane still remain unclear. The M proteins of the NDV are particularly important in providing the driving force that brings the assembled RNP core to the appropriate place at the plasma membrane to form a budding virion particle. It is suggested that the cytoplasmic tails of F and HN glycoproteins make important contacts with M proteins, which in turn associate with the nucleocapsid thus facilitating budding of the mature virions (Peeples, 1991). Figure 2.5 shows the life cycle of NDV.

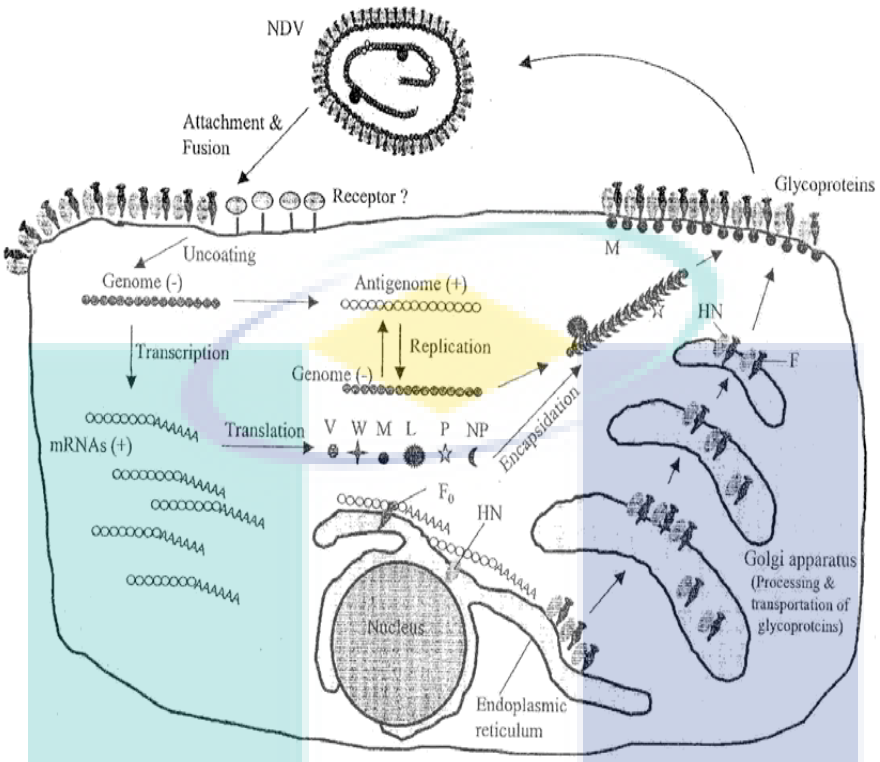

Figure 2.5: The life cycle of NDV. (Figure from review article by Yusoff, K., and W. S. Tan. 2001. Newcastle disease virus: macromolecules and opportunities. Av. Pathol. 30:439-455).

2.3 NEWCASTLE DISEASE VACCINES

Newcastle disease is highly contagious and attempts to control it by veterinary sanitary measures alone are unsatisfactory. Vaccination programs using vaccines are highly infective and cause minimum reactions to bird. Intensified vaccination programs have brought clinical Newcastle disease under control. Generally there are three types of commercially available vaccine for Newcastle disease: live lentogenic, live mesogenic, and inactivated vaccines (Allan et al., 1978).

2.3.1 Live lentogenic vaccines

Live lentogenic vaccines are usually produced from field viruses that have been shown to have low pathogenicity for poultry but produce an adequate immune response. Typical vaccine strains are Hitchner B1 (Hitchner and Johnson, 1948) and La Sota (Winterfield and Fadly, 1973) which are possibly the two most widely used animal vaccines and also F strain (Asplin, 1952) and V4. However, these viruses have frequently been subjected to selection pressures by manufacturers in order to improve their immunogenicity or to enable their use by a particular method of application.

Live lentogenic vaccines may be given to birds individually by eye drop or beak dipping but it is usually more practical to use methods of mass application such as in the drinking water or by machines generating sprays or aerosols. Aerosols have particular use during epizootics in the face of quickly spreading disease, as administration of lentogenic vaccines strains in this way may enable rapid vaccination of large number of birds and generally the immune response is particularly fast. However, spray and aerosols, particularly aerosols with small particle size that may penetrate deeply into the respiratory tract, result in reactions that will be most severe in fully susceptible birds. Use of aerosols of La Sota vaccine on such birds may result in heavy mortality (Pattinson et al., 2008). For the lentogenic vaccines, the optimum doses is usually considered to be between $10^{6.5}$ and $10^{7.0}EID_{50}$ per bird (Allan et al., 1978).

2.3.2 Live mesogenic vaccines

Mesogenic vaccines such as Roakin (Beaudette and Black, 1946), Mukteswar (Haddow and Idnani, 1946), Komarov (Komarov and Goldsmit, 1946), and Hertfordshire (Iyer and Dobson, 1940) have usually been derived in the laboratory from fully virulent strains. Their use is generally restricted to countries where there is a problem due to enzootic virulent viruses. Method of

application varies with strain. Some may be given in drinking water while others require intradermal inoculation via the wing web. Mesogenic vaccine viruses are capable of causing severe disease and must on be administered following primary vaccination with lentogenic viruses (Pattinson et al., 2008). For live mesogenic vaccines, the optimum dose is approximately 10^5 EID_{50} per bird (Allan et al., 1978).

2.3.3 Inactivated vaccines

Inactivated vaccines are usually prepared from egg grown virus that is killed by treatment with formalin or β-propiolactone. Aqueous inactivated vaccines have been used but in recent years these have been superseded by those based on oil. The immunogenicity of such vaccines may vary considerably with the type and ratios of the components of the vaccine. Both virulent and avirulent viruses have been used as a source of antigen for inactivated vaccines. From a safety aspect, viruses of low virulence would seem the most sensible source of antigen and have the added advantage of usually growing to higher titres in eggs (Allan et al., 1978; Pattinson et al., 2008).

2.4 ANIMAL CELL CULTURE

Cells when removed from animal tissue or whole animal will continue to grow if supplied with nutrients and growth factors. This process is called cell culture. It happens *in vitro* (‘in glass’) as opposed to *in vivo* (‘in life') (Butler, 2004). Generally, the term "cell culture" refers to the culturing of cells derived from multi-cellular eukaryotes, especially animal cells. Cell cultures are derived from dispersed cells taken from original tissue and disaggregated by enzymatic, mechanical, or chemical means (Freshney, 2004). The culture process permits single cells to act as independent units much like any microorganism such as bacteria or fungi. The cells able to divide by mitosis

and the cell population can continue growth until limited by some parameter such as nutrient depletion (Butler, 2004).

One of the earliest purposes of cell culture development was for the production of virus vaccines. Microorganisms such as virus are unable to be propagated in artificial media but requires live cell to grow (Sinha and Kumar, 2008). In 1955, an inactivated vaccine against polio disease was approved in the United States of America. The vaccine is produced on large scale by using primary monkey kidney cells (Alves et al., 2008). Later in 1960s, at the Wistar Institute in Philadelphia, Dr. Hayflick has developed a cell line from embryonic tissue capable of replicating more than 50-fold before becoming senescent (Hayflick and Moorehead, 1961). The cell was diploid, easy to freeze and to reactivate and did not show any evidence of contamination by the viruses normally found in monkey primary kidney cells. This cell line (WI-38) turned into the basis for the production of human viral vaccines against poliomyelitis and MMR (measles, mumps, and rubella), while other cell lines were evaluated for the production of animal vaccines, such as BHK (baby hamster kidney) in the case of the vaccine against foot-and-mouth disease (Alves et al., 2008).

Apart from production of human and animal vaccines, animal cell culture is also used in many other applications. Butler (2004) has listed several applications for animal cell culture:

i. To study the normal physiology or biochemistry of cells. For example, metabolic pathways can be investigated by applying radioactively labeled substrates and subsequently looking at products.

ii. To test the effects of compounds on specific cell types. Such compounds may be metabolites, hormones, or growth factors. Similarly, potentially toxic or mutagenic compounds may be evaluated in cell culture.

iii. To synthesize artificial tissue by combining specific cell types in sequence. This has considerable potential for production of artificial skin for use in treatment of burns.

iv. To produce valuable biological products (e.g. monoclonal antibodies and glycoproteins) from large-scale cell cultures.

2.4.1 Types of cell cultures

In theory, tissues or organ can be taken from any organism and grown *in vitro* to produce primary cell cultures. If desired, these primary cultures can be sub cultured. Some cultures especially with fetal origin can be serially passaged but will eventually reach senescence; termed as finite cell lines. Other cultures, which may spontaneously undergo crisis, be derived from tumor material, exposed to transforming agents and have the capacity to be subcultured indefinitely are termed as continuous cell lines (Blake and Stacey, 1999).

2.4.1.1 Primary cell culture

Primary cell culture refers to culture of cells obtained directly from the tissues or organs aseptically collected from different apparently healthy animals. From these tissues or organs, the cells can be disintegrated by mechanical, chemical or enzymatic digestion. When these cells are induced to grow *in vitro,* on suitable tissue culture media, cell cultures are formed and are called primary cell culture (Sinha and Kumar, 2008). Primary cultures are often established from embryonic tissue because the cells are more easily dispersed and have a superior growth potential. The structure of a tissue is highly ordered and comprises a range of cell types. Usually the main objective of establishing a primary culture is to select a single cell type from this ordered structure (Butler, 2004).

Primary cells *in vitro* grow as adherent monolayer on a solid substrate or as suspension in the medium. From one animal several bottles and flasks culture can be prepared (Sinha and Kumar, 2008). Although primary cultures have several major drawbacks (e.g. time-consuming preparation, require use of live animals or fresh tissue, low growth rate, prone to contamination etc.), in several cases they are still preferred (Mather and Roberts, 1998). This is because since they are recently removed from the *in vivo* situation, they are more representative of the cell types in the tissue from which they were derived and in the expression of tissue-specific properties (Freshney, 2000). There are generally five main types of animal tissues from which cell cultures are usually derived (Butler, 2004):

i. Epithelial tissue consists of a layer of cells which cover organs and line cavities; examples include skin and the lining of the alimentary canal. The epithelial cells grow well in culture as a single cell monolayer and have a characteristic cobble-stone appearance.

ii. Connective tissue forms a major structural component of animals, consisting of a fibrous matrix and including bone cartilage. The tissue contains fibroblasts which are amongst the most widely used cells in laboratory cultures. Fibroblasts are bound to the fibrous protein collagen in the connective tissue. The cells are spherical when first dissociated by trypsin from the tissue but elongate to a characteristic spindle-shape on attachment to a solid surface. Fibroblasts have excellent growth characteristics and have been the 'favorite' cells for establishing cultures. Fibroblast and epithelial cells adapt relatively easily to culture and have growth rates with a doubling time of 18–24 hours.

iii. Muscle tissue consists of a series of tubules formed from precursor cells which fuse to form a multinucleate complex and which also contain the structural proteins (actin and myosin). The precursor cells are myoblasts which are capable of differentiation to form myotubes - a process that can be observed in culture.

iv. Nervous tissue consists of characteristically shaped neurons which are responsible for the transmission of electrical impulses and supporting cells, such as glial cells. Neurons are highly differentiated and have not been observed to divide in culture. However, the addition of nerve growth factor to cultures of neurons may cause the formation of cytoplasmic outgrowths called neurites. Some of the characteristics of nerve cells can be observed with neuroblastomas which are tumor cells that undergo cell growth in culture.

v. Blood and lymph contain a range of cells in suspension. Some of these will continue growth in a culture suspension. These include the lymphoblasts which are white blood cells and are used extensively in culture because of their ability to secrete immune-regulating compounds.

2.4.1.2 Finite cell lines

After successive subcultures of a very heterogeneous primary culture, containing many cell types of the original tissue, a more homogeneous cell line with a higher growth rate may arise. A finite cell line can be serially propagated in culture, usually for only a limited number of cell divisions. Finite cell lines are generally diploid and maintain some degree of differentiation (Leo et al., 2008). Nevertheless, these cell lines die after a limited number of generations, the Hayflick limit, which is usually about 30–50 division cycles depending on the origin of the cells (Hayflick and

Moorehead, 1961). Examples of finite cell lines include widely used cells such as WI-38 and MRC-5. Finite cell lines originally derived from embryonic tissues will generally have the potential for a greater number of population doublings before the onset of senescence than those derived from adult tissues (Blake and Stacey, 1999).

2.4.1.3 Continuous cell lines

Continuous cell lines include transformed and immortalized cell lines and are derived directly from tumor material or by exposure of cells due to transforming agents. Transformation of cells may be due to: (i) exposure to chemical carcinogens; (ii) ionizing radiation; (iii) infection with retroviruses or DNA tumor viruses (or viral components); (iv) 'spontaneous' (Blake and Stacey, 1999). Cells that undergo transformation have the capability of an unlimited number of cell division *in vitro* as long as they are supplied with enough nutrients.

According to Freshney (2000), the main characteristics of continuous cell lines are: (i) altered cell morphology (smaller, less adherent, or more rounded cells, with a higher nucleus to cytoplasm ratio); (ii) higher growth rate (duplication times decrease from 36–48 hours to 12–36 hours); (iii) less dependency on blood serum or selected growth factors; (iv) increase in cloning efficiency; (v) increase in heteroploidy (chromosomal variation between cells) and in aneuploidy (divergence from the original diploid number); (vi) increase in tumorigenicity. Although continuous cell line provides unlimited cell supply, it also has several disadvantages: (i) greater chromosomal instability; (ii) loss of tissue specific markers; (iii) divergence from the donor phenotype. Examples of continuous cell lines are Vero cells, obtained from African green monkey kidney, HeLa cells, obtained from a human carcinoma and CHO cells from chinese hamster ovary (Sinha and Kumar, 2008).

2.4.1.3.1 Vero cell line

Vero cell is an aneuploid and continuous cell line derived from kidney epithelial cells of the African green monkey. The cell line was founded by Y. Yasamura and Y. Kawakita at the Chiba University in Japan on March 27, 1962 (Yasamura and Kawakita, 1963).

Apart from virology studies, this anchorage-dependent cell line also has been used in many other applications, including the propagation and study of intracellular bacteria (e.g., Rickettsia) and parasites (e.g., Neospora), assessment of the effects of chemicals, toxins and other substances on mammalian cells at the molecular level (Ammerman et al., 2008) and cytotoxicity studies of biomaterials projected for repairing or reconstituting injured human tissues (Leo et al., 2008). Furthermore, Vero cells have been licensed in the United States for production of both live (rotavirus, smallpox) and inactivated (poliovirus) viral vaccines. Throughout the world Vero cells have been used for the production of a lot of viruses, including Rabies virus (Trabelsi et al., 2005), Reovirus (Berry et al., 1999), avian inlfluenza virus (Satterlee, 2008) and Japanese encephalitis virus (Toriniwa and Komiya, 2008).

The reason behind the use of Vero cells rather than diploid cells is that the cells are readily adapted for growth in bioreactors on microcarriers and provide consistently higher yield of viruses. Production of virus using Vero cells provides greater vaccine purity (less contaminating cell debris), larger lots of vaccine and more economic production of vaccine (Duchene et al., 1990).

2.4.2 Mode of culture

2.4.2.1 Anchorage dependent cell culture

Adherent cells which are also known as anchorage-dependent cells require attachment to a substratum for their proliferation. They are generally subject to contact inhibition, i.e., they grow

as an adherent monolayer and stop dividing when they reach a density that they touch each other. Most cells with exception of mature hematopoietic cells and transformed cells grow in this way (Sinha and Kumar, 2008). At the laboratory scale the substratum can be provided by the solid surface of Petri dishes, T-flasks, or Roux bottles which are made of specially treated glass or plastic.

The interaction between the cell membrane and the growth surface is critical and involves a combination of electrostatic attraction and van der Waal's forces (Butler, 2004). Cells bind to surface through specific cell surface receptors to attachment proteins absorbed to the surfaces (Sinha and Kumar, 2008). Two factors in culture medium essential for adhesion of cell to culture surface are divalent cations (usually Ca^{2+}) and protein (Grinnell 1978). The protein molecule essential for full adhesion of cells to a culture surface is known to be a glycoprotein (Yamada and Olden, 1978). In the absence of divalent cations and proteins, cells attach to a culture surface only by a nonspecific adsorption (Grinnel et al., 1977). Serum provides some of these adhesion factors that are essential components of extracellular matrix. Glycoprotein is found in the serum in culture medium as cold insoluble globulin or is secreted from certain cells as fibronectin (Hughes et al., 1979).

In addition, the density of the electrostatic charge on the solid substratum is also critical in maximizing cell attachment. A negative charge is provided on glass surface containers by alkali treatment. Tissue culture-grade plasticware consists of sulfonated polystyrene with a surface charge of 2–5 negatively charged groups per nm^2 (Butler, 2004).

2.4.2.2 Suspension cell culture

Suspension cultures are obtained from cells that can survive and proliferate without attachment to the substratum (anchorage independent). Examples of this cell type are transformed cell lines, cells from malignant tumors and cells of hematopoietic origin (Freshney, 2005). These cell lines do not produce attachment factors and remain in suspension either as single cells or as clumps of cells. Lymphoblastoids and many other tumour cells do not require a surface on which to grow and therefore do not require proteases such as trypsin for subculturing (Meleady and O'Connor, 2006).

Suspension cultures present some advantages in comparison with adherent cell cultures. Large cell quantities can be attained without increasing the superficial area of the substratum. Also, a steady state can be reached if nutrients are continuously supplied and cell concentration is kept constant. This can rarely be achieved with a monolayer culture (Leo et al., 2008).

The main disadvantage of suspension culture lies in its technique of subculturing which is by dilution. During growth, cells produce metabolic by-products, which become toxic if allowed to accumulate in the medium. Initially, sufficient waste metabolites will be removed by diluting cell cultures, allowing growth to continue but the viability of the cells will gradually decline after a few subcultures. Surviving cells may also undergo selection pressures, resulting in altered characteristics. Subculture by sedimentation overcomes this problem of toxic metabolites but one must be careful not to over dilute the cells, which will increase the lag phase of the cells and may prevent them from dividing and also removes any growth factors produced by the cells (Meleady and O'Connor, 2006).

2.4.3 The phases of culture

Normal cells in culture show a sigmoid pattern of proliferative activity that reflects culture adaptation, environmental conditioning, nutrient availability and for adherent cells, available free adhesion surface (Leo et al., 2008). When cells are inoculated into a vessel firstly they will enter a period named lag phase, followed by a period of exponential growth (the 'log phase'), and finally enter a period of reduced or zero growth (decline phase) after they become confluent ('plateau or stationary phase') (Figure 2.5). These phases are distinct for each cell line and give rise to measurements which should be reproducible with each serial passage: the length of the lag period, the cell doubling time (t_d) in mid-log phase, and the saturation density at plateau, given that the environmental conditions are kept constant (Freshney, 2000). Cell growth can be mathematically represented by the following general equation:

$$\frac{dX}{dt} = \mu.X \qquad (2.1)$$

where μ refers to specific cell growth rate, X is cell concentration and t is the culture time.

2.4.3.1 Lag phase

This is an early phase in which there is no apparent increase in cell concentration. During this phase cells produce growth factors which may be required to reach a critical concentration before growth takes place (Butler, 2004). It is an adaptation period in which adherent cells resynthesize the glycocalyx elements lost during trypsinization, bind, and spread on the substratum. During spreading, the cytoskeleton reappears, and new structural proteins are synthesized. The activity of enzymes such as DNA polymerase increases followed by the synthesis of new DNA and structural

proteins. Some specialized cell products may disappear and not reappear until the cessation of cell proliferation at a high cell density (Freshney, 2005).

The duration of the lag phase is dependent on at least two factors: the point in the growth phase from which cells were taken in the previous culture and the inoculum concentration. The lag phase tends to become longer when the inoculum is from lag, stationary or decline phase. On the other hand, cells may multiply without lag phase if taken from log phase. Furthermore, cultures initiated at low cell concentrations also increase the duration of the lag phase (Leo et al., 2008).

2.4.3.2 Exponential phase

This phase which is also called as log phase is the period of exponential increase in the cell number following the lag period and terminating one or two population doublings after confluence is reached (Freshney, 2005). During this phase cells undergo change in biochemical and respiratory activity (Sinha and Kumar, 2008). The percentage of cells in division can reach 90–100%, and cells are in their best physiological state, which is ideal for cell function studies. This phase is characterized by cell doubling time, (t_d) which is a kinetic profile typical for all cell lines. t_d can be determined through the direct integration of equation (1), since μ is constant and, in this phase, attains a maximum value (μ_{max}), resulting in:

$$t_d = \left(\frac{\ln 2}{\mu_{max}}\right) \qquad (2.2)$$

Factors that influence the duration of the log phase are inoculum concentration, cell growth rate, nutrient availability, and accumulation of inhibitory metabolites. For adherent cells, the end

of the log phase may also occur at confluence, when cells cover the entire available growth surface, at which point contact inhibition restricts further growth (Leo et al., 2008).

2.4.3.3 Stationary phase

During the stationary or plateau phase, cell growth rate is reduced due to reduced cell spreading (Folkman and Moscona, 1978), buildup of inhibitory metabolites, and depletion of nutrients, particularly growth factors in the medium (Dulbecco and Elkington, 1973). In some cases, proliferation almost completely ceases. During this phase, cell division is equilibrated with cell death, and the percentage of cells in division falls to between 0 and 10%. At confluence (i.e. all of the available growth surface is occupied and all of the cells are in contact with surrounding cells) the growth of adherent cells is inhibited by cell to cell contact but a certain degree of mitotic activity may still be observed. Cells occupy a smaller surface area, exposing less of their own membrane surface to the culture medium. A relative increase in specialized protein synthesis (as opposed to structural proteins) as well as a change in cell surface composition and charge modification may occur (Freshney, 2005). The stationary phase may be prolonged if the culture medium is replenished with fresh medium. This is not a stable period for most cell lines, and they are more susceptible to injuries (Leo et al., 2008).

2.4.3.4 Decline phase

In this phase there is rapid decline in the number of cells as division of cells practically ceases as nutrients are completely exhausted and there is accumulation of inhibitory metabolites (Sinha and Kumar, 2008). Cell death can occur by two distinct mechanisms, named necrosis and apoptosis. Necrosis occurs because of an irreversible injury and normal homeostasis is lost. Apoptosis, on

the other hand, occurs through the activation of a biochemical program involving a cascade of cell components, which is internally controlled and requiring energy (Leo et al., 2008).

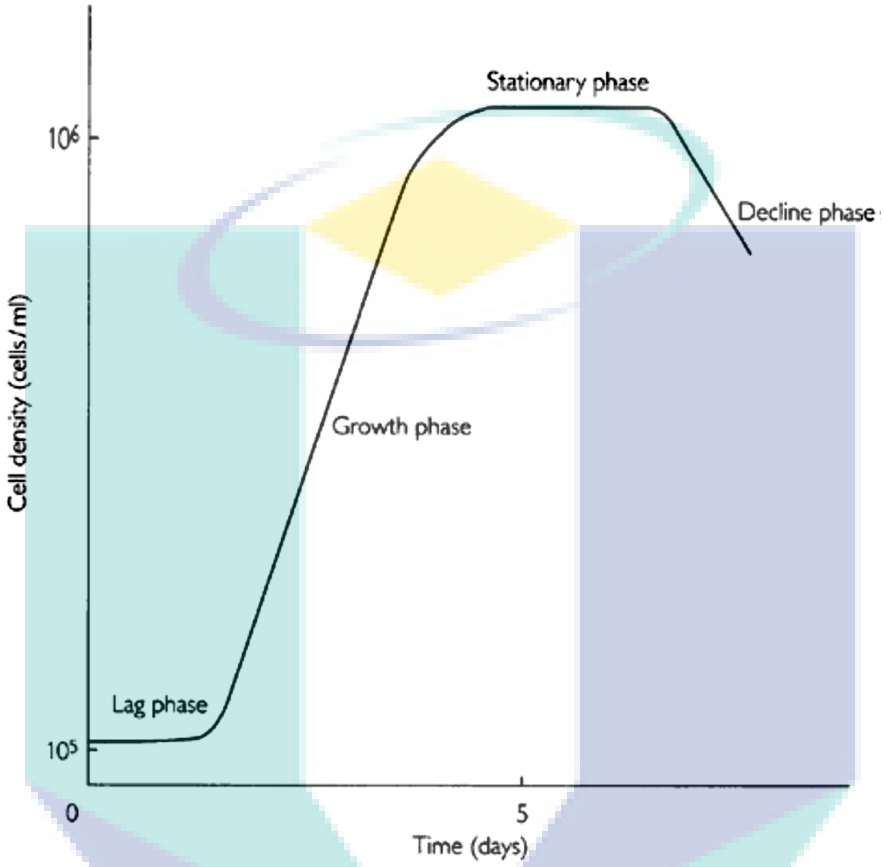

Figure 2.6: Phases of growth in cell culture (Butler, 2004).

2.4.4 Bioreactor for animal cells

Small volume cell cultures are preferable for experiments requiring a large number of replicates. This might be necessary when use in the determination of the concentration-dependent effects of substances that promotes or inhibits cell growth. However, if cell cultures are intended for producing substantial quantities of a cell product, such as a virus or a glycoprotein, or to obtain a sufficient quantity of cells for metabolite or enzyme extraction, small volume cell cultures no longer makes sense. Culture scale up is required and in animal cell culture it can be provided by

the use of bioreactor system units which generally has volume capacity of 500 ml up to thousands of liters (Butler, 2004). The bioreactors used for animal cell culture can be classified according to different criteria. One of the most useful refers to the homogeneity of the system. All culture systems are multiphase systems, since at least two phases are involved: a solid one (cells) and a liquid one (culture medium). Bioreactors in which cells are uniformly suspended in the liquid phase are designated homogeneous bioreactors. On the other hand, those bioreactors in which cells are not homogeneously distributed in the entire volume are called heterogeneous bioreactors (Veliz et al., 2008).

2.4.4.1 Homogeneous bioreactors

Several types of homogeneous bioreactors used in animal cell culture are spinner vessel, stirred-tank, airlift, and wave bioreactors. One of the most important advantages of using homogeneous bioreactors is the possibility of monitoring and controlling the physicochemical environment to which the cells are submitted in a much more reliable way than in heterogeneous bioreactors. Thus, it is possible to indirectly control the physiological state of cells (Veliz et al., 2008).

2.4.4.1.1 Spinner vessel

Spinner vessel is a straight-sided glass flask containing a suspended central Teflon paddle containing a magnet, which turns and agitates the culture when placed on a magnetic stirrer. The stirring normally stable over a long period at a rotation speed of between 10 and 300 rpm. The vessel is usually fitted with one or more side arms that are useful for sampling or as ports for probes or tubing. Spinner vessel can be designed up to a capacity of 10 liters. Cultures above this volume need a top-driven motor for stirring. Spinner vessel is suited for growing suspension cells

although they can be adapted for anchorage-dependent cells by the use of microcarriers. Spinner vessel is usually siliconized to prevent undue attachment of cells to the inner glass surface. This may be performed by application of dimethyldichlorosilane (Repelcote from Sigma) (Butler M., 2004).

2.4.4.2 Heterogeneous bioreactors

In this type of bioreactor, there is a compartment where cells remain attached to a surface or immobilized on or inside a biocompatible bed. Culture medium has to be pumped through this compartment for cells to have access to nutrients and dissolved oxygen. The main disadvantages of these bioreactors, developed for the cultivation of adherent cells that require a surface for proliferation, are the impossibility of obtaining homogeneous samples of the cell population and the limitations of scale-up. Examples of heterogeneous bioreactors used in culture of animal cells are packed bed, fluidized bed, stacked plate, and hollow fiber bioreactors (Veliz et al., 2008).

Packed bed bioreactor consists of a static support matrix for the attachment and growth of anchorage-dependent cells. Cell growth is supported by a continuous flow of culture medium (Butler, 2004). Culture medium is recirculated through the packed bed, promoting transfer of nutrients, metabolites, and products. Before being pumped into the bed, the culture medium should be enriched in dissolved oxygen. This can be accomplished by vigorous stirring in aeration tanks or by the use of membrane modules, generally positioned in an external recirculation loop. The size of the packed bed is a critical parameter, since the dissolved oxygen concentration in the medium gradually decreases as the liquid flows through the bed (Fassnacht and Porter, 1999). One of the advantages of this system is that a clarified fluid is obtained at the bioreactor outlet, with no need for a cell separation device (Veliz et al., 2008).

2.4.4.3 Microcarrier cell culture technology

Microcarriers are small particles, made of materials such as cellulose, dextran, glass, collagen, or gelatin. Microcarriers are designed to provide a pseudo-suspension culture for anchorage-dependent cells (Butler, 1987). They are microscopic-sized particles that are easily maintained in suspension in liquid medium. Generally, they have a spherical shape and present a surface structure and composition that promotes cell adhesion and growth (Veliz et al., 2008).

Most microcarriers have a bead diameter of 90–300 µm. The surface is charged so as to ensure cell attachment. The density of the electrostatic charge on the microcarrier surface is critical to allow cell attachment and growth. The cells will then grow over the outer surface area until a monolayer is formed. Each microcarrier will accommodate 100–200 cells. It is important at inoculation that there is an even distribution of cells on the available microcarriers so that there are no 'empty' microcarriers at the end of the growth period. The density of a microcarrier is approximately 1.02–1.04 g/cm^3, which is slightly higher than that of the culture medium. Thus, they can be maintained in suspension at low agitation speeds. Also, it is important to ensure an agitation rate that allows the microcarriers to be suspended in the culture medium but does not cause damage to the cells (Butler, 2004). Furthermore, since their diameter is relatively large, the carriers settle down easily when agitation is stopped, facilitating harvesting (Veliz et al., 2008). Cells can be detached from microcarriers by proteolytic enzymes (e.g. trypsin or collagenase) although, for processes that are designed for the production of a secreted cell product, cell removal may be unnecessary (Butler, 2004).

The first culture of cells on microcarriers was developed by van Wezel in the 1960s (van Wezel, 1967), using DEAE-Sephadex^{TM} 50 gel beads originally designed as ion-exchange chromatography beads. The first product to be produced industrially using microcarriers (an

inactivated polio vaccine) was developed by van Wezel himself several years later. Nowadays, the main industrial use of microcarriers is in the production of vaccines, but also of gene therapy vectors, recombinant proteins, and monoclonal antibodies. In the literature, there are reports of culture scale-up to 6000 L for Vero cells cultivated on Cytodex^{TM} carriers (GE Healthcare, 2006).

2.5 VIRUS CULTURE

Viruses are obligate intracellular parasitic microorganisms that require living cells in order to replicate. Generally, viruses are cultured for two main reasons; the first is for the diagnostic identification of agents associated with disease. The other is to enable some subsequent experimental manipulation of the virus to be performed, for example, to examine mechanisms of replication or to determine the effectiveness of potential antiviral strategies *in vitro* (Cann and Irving, 1999).

Several techniques have been developed for the culture of viruses in cell-free systems, but in the vast majority of cases it is necessary to supply the virus with appropriate cells in which it can replicate. Phages are supplied with bacterial cultures, plant viruses may be supplied with specially cultivated plants or with cultures of protoplasts (plant cells from which the cell wall has been removed), while animal viruses may be supplied with whole organisms, such as mice, embryonated chicken eggs or insect larvae. For the most part however, animal viruses are grown in animal cell culture (Carter and Saunders, 2007).

2.5.1 Virus cultivation in embryonated chicken eggs

The technique of using embryonated chicken eggs was first established by an American pathologist named Ernest William Goodpasture in 1931 (Goodpasture et al., 1932). Embryonated chicken eggs

are among the most useful and available forms of living animal tissue for the isolation and identification of animal viruses, for titrating viruses, and for quantity cultivation in the production of viral vaccines. The optimal age of egg for virus inoculation is 10 days of incubation. Eggs of this age provide variety of differentiated tissues including chorioallantoic membrane, yolk sac, allantoic sac, or amniotic sac which can serve as substrates for growth of a wide variety of viruses, including orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, herpesviruses, and poxviruses (Kuchler, 1977). Eggs of 8 days or less embryonation tend to be more susceptible to nonspecific death and provide a harvest of allantoic fluid which is low in virus titer (Gallili and Nathan, 1998). Several viruses when introduced onto the chorioallantoic membrane can cause distinct and characteristic foci, thus providing a method for identification of virus types, or for quantifying virus stocks or assessing virus pathogenicity (Kuchler, 1977).

Culturing of viruses using embryonated chicken eggs is highly susceptible to contamination. One of the main sources of contamination is the eggs themselves and care should be taken to fumigate them effectively before their introduction into the inoculation room. Egg shell surfaces are generally contaminated with a variety of microorganisms; thus, after continual use the egg incubators also become contaminated. Incubators should be fumigated regularly with formaldehyde gas to remove all the microorganisms that can cause contamination (Allan et al., 1978). To reduce further the level of contamination, eggs used for culturing viruses also should be obtained from a specific pathogen free (SPF) flock and their transmission to the virus production area must be done under strict hygienic conditions (Gallili and Nathan, 1998).

All strains of Newcastle disease virus can replicate in embryonated chicken eggs. Embryonated chicken eggs should be obtained from SPF flock and incubated for 9-10 days at 37°C before use. If SPF eggs cannot be obtained, eggs from a flock free of NDV antibodies should be

used. Newcastle disease virus strains and isolates vary in their capacity and time taken to kill chick embryos. Virus titers are also influenced by strain, with the highest titers obtainable by those causing slow or no death (Gough et al., 1974). With some strains, embryo death and virus growth is affected by the presence of maternal antibodies in the yolk (French et al., 1967).

The route of inoculation is also important (Beard and Hanson, 1984). Inoculation of NDV via the yolk sac, as compared with the allantoic cavity, produced more rapid embryo deaths and caused deaths by strains that do not consistently kill by the latter route (Estupinan et al., 1968).

2.5.2 Virus cultivation in cell culture

Cultured cells currently provide the most widely used and most powerful hosts for cultivation and assay of viruses (Condit, 2001). The critical transition from using animal to cell culture for virus cultivation came into four important steps: (a) Sanford at the National Institutes of Health (NIH) overcame the difficulty of culturing single cells (Sanford et al., 1948), (b) George Gey and his colleagues at Johns Hopkins Medical School cultured and passaged human cells for the first time and developed a line of cells (HeLa) from a cervical carcinoma (Gey et al., 1952), (c) Harry Eagle at the NIH developed an optimal medium for the culture of single cells (Eagle, 1955), and (d) in a demonstration of the utility of all this, Enders and his colleagues showed that poliovirus could replicate in a non-neuronal human explant of embryonic tissues (Enders et al., 1949).

These ideas, technical achievements, and experimental materials had two immediate effects in virology. First, they led to the development of the polio vaccine as the first vaccine produced by using cell culture. Poliovirus was grown in monkey kidney cells incubated in flasks (Hilleman, 1992). Second, the exploitation of cell culture for the study of viruses began the modern era of molecular virology. The first plaque assay for an animal virus in culture was with poliovirus and

it led to an analysis of poliovirus every bit as detailed and important as the contemporary work with bacteriophages (Dulbecco and Vogt, 1953).

Despite all the achievements, there are still void in cultivation of viruses using cell culture because not all viruses are able to infect and replicate in cells and some can only be grown in certain types of cell. Thus, a laboratory which intends to isolate a range of viruses from clinical material must have available a corresponding variety of cell substrates. In practice, most laboratories will run only two or three different cell types, each of which will support the replication of as broad a range of viruses as possible. Examples are primary monkey kidney cells, human embryonic fibroblast, Vero cells, Hep-2 cells and HeLa cells (Cann and Irving, 1999).

Virulent Newcastle disease virus strain can be propagated in many cell culture systems, and viruses of low virulence can be induced to replicate in some of them. It is possible to use primary cell cultures or even cell lines for routine isolation of NDV. Lancaster (1966) has listed 18 primary cell types and 11 cell lines as susceptible. Many more have been added to the list since his 1966 report.

CHAPTER THREE

MATERIALS AND METHODS

3.1 INTRODUCTION

In this chapter, the experimental materials and methods are described for the production of Newcastle disease virus and UVO treated PS microcarriers. An overview of the experimental studies is shown in Figure 3.1. The analytical methods to determine the amount of carboxyl groups, functional groups, surface morphology, cell number, virus infectivity are presented in detail. The equipment, samples, chemicals and reagents, microorganisms, and media composition are described in the experimental material section.

3.2 MATERIALS

The major experimental materials are polystyrene powder, ND virus strain and Vero cell line. Other chemical and reagents, equipment and machineries, glassware and various consumable items are described in the following sections.

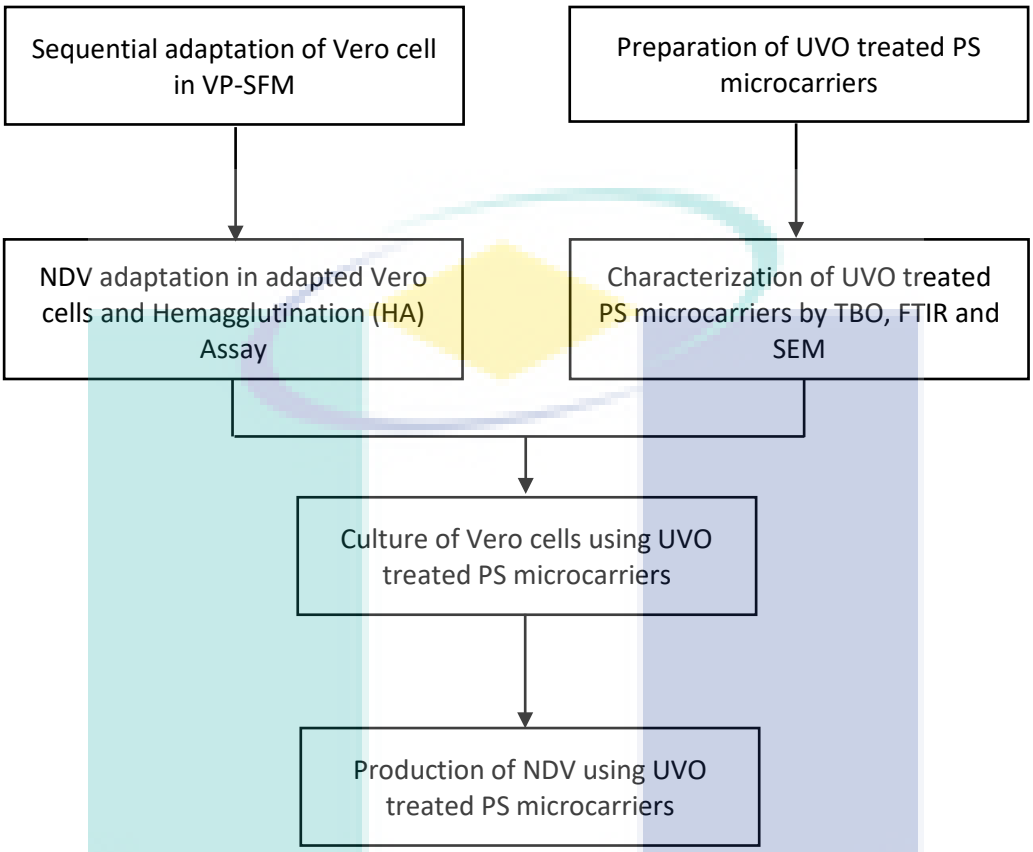

Figure 3.1: An overview of experimental studies.

3.2.1 Virus Strains

Mesogenic vaccine strain of Newcastle disease virus, La Sota was obtained from Universiti Putra Malaysia (UPM).

3.2.2 Cell lines

Vero cell line (ATCC-CCL-81™) was obtained from the American Type Culture Collection (ATCC).

3.2.3 Culture medium

Two different culture medium, Dulbecco's Modification of Eagle's Medium, DMEM (with glucose and L-glutamine) and Virus-Production Serum Free Medium (VP-SFM) were supplied by Gibco® in powder form.

3.2.4 Bioreactor Systems

Two different bioreactors systems were used in this research; T-flasks (25 cm^2 with 5 ml working volume, 75 cm^3 with 15 ml working volume, and 150 cm^3 with 30 ml working volume) and spinner flask (Medigene, 500 ml with 200 ml working volume).

3.2.5 Chemicals and reagents

Fetal Bovine Serum (FBS), Trypsin-EDTA, Accutase, Pen-Strep and Trypan Blue were obtained from Essen-Haus SDN. BHD (Malaysia). Fetal bovine serum (Gibco), antibiotics (100 U/ml penicillin, 0.1 g/l streptomycin) (Gibco), trypsin (Gibco), EDTA (Gibco), trypan blue (Gibco), phosphate buffered saline (Ginco), sodium bicarbonate, (Merck), ethyl acetate (Merck), hydrochloric acid (HmbG Chemical), sodium hydroxide (Merck) and ethanol 95% (Fischer Scientific).

Polystyrene (PS) powder (250 µm, homopolymer) was purchased from Goodfellow (UK). Polyvinyl alcohol, PVA (MW=30 000, hydrolysis degree≥85.0%) and chloroform were obtained from Merck (Germany). Oxygen gas was supplied by Linde (Malaysia). Glutaraldehyde, sodium cacodylate and osmium tetroxide were purchased from Agar Scientific (UK). Acetone, toluidine blue O (TBO), sodium dodecyl sulfate (SDS) and other chemicals were supplied by Sigma (USA).

3.2.6 Equipment

Various equipment and instruments used throughout the study were UVO treatment chamber, NANO Ozone Generator, overhead stirrer, biosafety cabinet, humidified CO_2 incubator, oven, drying cabinet, autoclave, inverted phase microscope, hot plate, magnetic stirrer, automatic pipette, pH meter, fume hood, measuring balance, water bath, centrifuges etc.

3.2.7 Consumable items

Consumable items such as Erlenmeyer flasks, graduated cylinders, glass beakers, centrifuge tubes, microtiter plates, hemocytometer chamber, glass rod, spatula, forceps, autoclave tape, autoclave bag, parafilm 'M', disposable serological pipettes, disposable media filtration units, cryovial tubes, syringes etc. were used in the present study.

3.3 MICROCARRIER PREPARATION AND ANALYSES

3.3.1 Preparation of polystyrene (PS) microspheres

Polystyrene (PS) microspheres were produced by oil-in-water (o/w) solvent evaporation method adapted from Zhu et al. (2002). Firstly, the oil phase was prepared by dissolving 60 g of PS in 300 mL of chloroform at room temperature. Next, the resulting clear solution was added drop-wise into 2 liters of 1% poly(vinyl alcohol) (PVA) solution (aqueous phase) at 80˚C and agitated at 330 rpm. Agitation was continued for 6 hours to completely evaporate the chloroform. Microspheres were collected by vacuum filtration, washed several times with distilled water and dried at 50°C overnight. Dried microspheres were sieved to collect between 150 and 200 µm microspheres and were later stored in a cool dry place until further use.

3.3.2 Ultraviolet/ozone (UVO_3) treatment

A 250 mL conical flask (borosilicate glass) containing 5 g of PS microspheres was fixed on a shaker and shaken at 250 rpm for homogenization. PS microspheres were then aerated with ozone at a concentration of 77 728.24ppm (in O_2 flowrate of 0.5 lpm) and irradiated from both sides with four 20 W low pressure mercury lamps (UV lamps) at λ=254 nm. Ozone was produced by NANO Ozone Generator (Absolute Ozone, Canada) while the radiation from UV lamps to the sample was measured to be at 0.343 $mWcm^{-2}$. Samples were treated by the UVO system for 60 min. The setup of the UVO system used in this work is presented in Fig. 3.2.

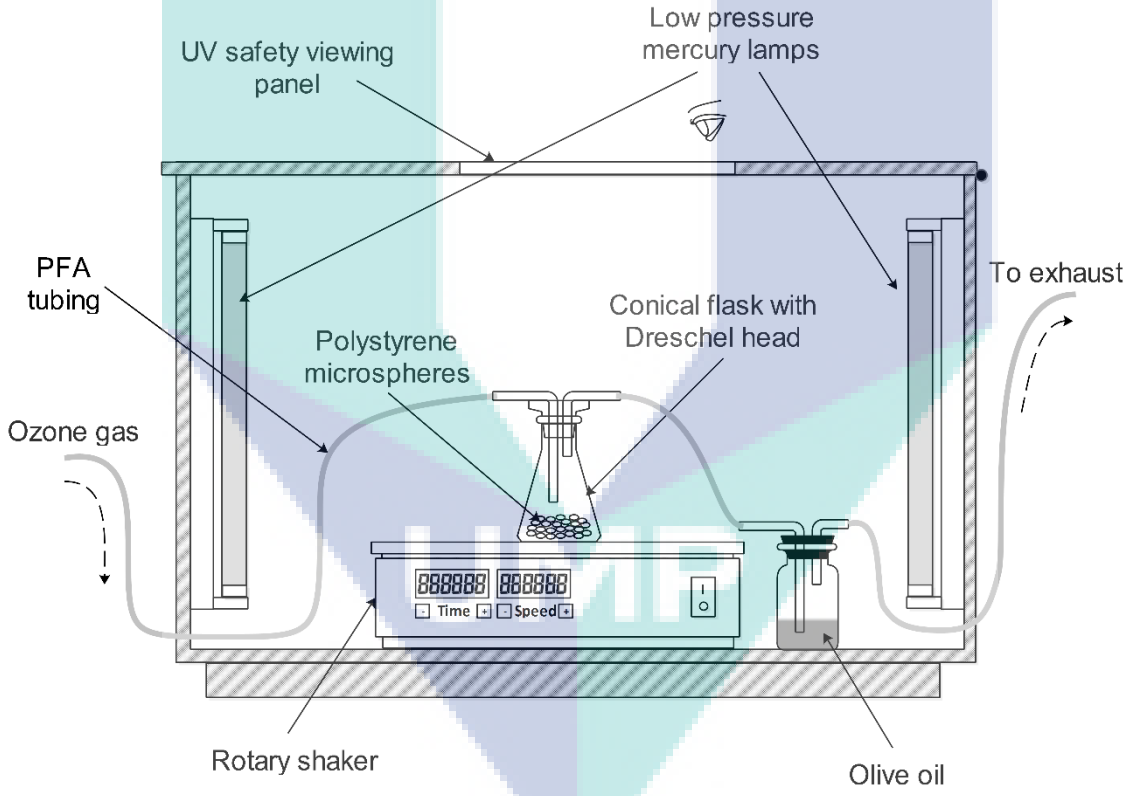

Figure 3.2: Assembly of the ultraviolet/ozone (UVO_3) system

3.3.3 Determination of carboxyl groups

The concentration of carboxyl (COOH) functional group introduced onto PS microspheres was measured by toluidine blue O (TBO) assay. The method was adapted from Rödiger et al. (2011) with some modifications. Briefly, 1 g of microspheres were incubated in 10 mL of toluidine blue O (TBO) solution (1 mM NaOH, 0.1% TBO) for 30 minutes at 40°C and were shaken at 400 rpm. Next, microspheres were washed thoroughly with 1 mM NaOH solution using vacuum filtration until the rinsing solution become colorless. TBO bound on microspheres were later desorbed by incubation of microspheres in 10 mL of 20% SDS solution (30 minutes, 40°C, shaken at 400 rpm). Subsequently, microspheres were pelleted by centrifugation at 2250×g for 15 minutes. TBO absorption of SDS supernatants was measured at 625 nm with Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, USA). The amount of COOH on the surface of PS microspheres was determined by referring to a standard curve. Calculations were based on the assumption that TBO were bonded to equivalent moles of carboxylic groups (TBO/COOH = 1:1).

3.3.4 FTIR analysis

FTIR spectroscopy was used to examine the surface chemistry of PS microspheres, pre- and post-UVO_3 treatment as well as after gelatin immobilization. Surfaces were examined directly via attenuated total reflection-infrared (ATR/IR) method in the 550-4000 cm^{-1} region using Nicolet iS50 FTIR spectrometer (Thermo Scientific, USA). Analysis of transmission spectra was carried out using OMNIC software.

3.3.5 Sample imaging

PS microspheres were sprinkled on double sided adhesive tapes attached to aluminum stub. They were then sputter coated with thin layer of gold using Hitachi E-1010 Ion Sputter (Japan). The surface morphology of PS microspheres were examined and photographed by Hitachi S3400N Scanning Electron Microscope (Japan) at 15 kV. In conjunction with SEM, energy-dispersive X-ray spectroscopy (EDX) was also performed to determine the surface elemental composition of PS microspheres.

To observe the morphology of cells attached to the microcarriers, cell-microcarrier aggregates were fixed using 4% glutaraldehyde at 4 °C for 12 hours. Samples were washed three times (10 minutes for each washing) using 0.1 M sodium cacodylate. A second fixation was carried out with 1% osmium tetroxide at 4 °C for 2 hours and were re-washed with 0.1 M sodium cacodylate for three changes of 10 minutes each. After that, samples were dehydrated through series of ethanol solution with concentrations of 5%, 50%, 75%, 95% and 100%, respectively followed by critical point drying using Bal-Tec CPD030 Critical Point Dryer (Bal-Tec, Germany). Dried samples were sputter coated with gold and examined with scanning electron microscope.

3.4 BIOLOGICAL PREPARATION AND ANALYSES

3.4.1 DMEM preparation

Culture medium was prepared by first adding a packet of medium powder and desired amount of sodium bicarbonate, $NaHCO_3$ salts into a 1000 ml Schott bottle. Then it was followed by the addition of deionized water and then the mixture was stirred until the powder and salts completely dissolved. After that, the pH of the liquid medium was adjusted to 7.0-7.2 by using diluted acid and base (1M HCL and 1M NaOH). Next, 1% antibiotics (100 U/ml penicillin, 0.1 g/L

streptomycin) or penstrap was added into the liquid medium to reduce risk of contamination. The liquid medium was then transferred into biosafety cabinet for sterile filtration. Medium was poured into the receiving end of bottle top media filtration unit (equipped with 0.2 µm pore size PTFE membrane) which was prefixed with 1000 ml Schott bottle and vacuum pump. After filtration was done, the sterility of the medium was tested by incubating 5 ml of the prepared media using 25cm^3 T-flask in the humidified CO_2 incubator for few days. The remaining of the liquid medium was placed inside the chiller at 4ºC temperature.

3.4.2 VP-SFM preparation

VP-SFM was prepared with 17.6 g of VP-SFM AGT, 10 ml of Pen-Strep and 990 ml water, mixed and filtered with filtration kit of 0.2 µm pore size membrane filtration. 4mM of L-glutamine was added into VP-SFM prior to use in cell growth and adaptation.

3.4.3 Thawing of Cryopreserved Cells

Thawing was carried out as described by Freshney (2005). Frozen or cryopreserved cells in cryovial tubes were stored in liquid nitrogen and when taken out must be immediately thawed and cultured to prevent cell death. Frozen cells were thawed by rubbing the cryovial tubes with palms or by swirling in 37ºC water bath and immediately transferred into a centrifuge tube with a sterile pipette. Before adding the cells, culture medium supplemented with 10% fetal bovine serum (FBS) was added into the centrifuge tube. The cells were then centrifuged at 150g at 25ºC for 15 minutes. The supernatant was discarded and 10 ml of fresh medium supplemented with 10% FBS was used to resuspend the pellet. The cells were reseeded into a T-flask and later incubated at 37ºC in a humidified CO_2 incubator.

3.4.4 Subculturing

When cell becomes confluent or when growth media is exhausted, the cell were subcultured. Subculture was carried out as described by Butler (2004). Subculture was started by examining the culture in the T-flask under microscope. If the culture was confluent and there were no signs of contamination or deterioration, then the subculture procedures were resumed. In a 25cm^2 T-flask, used media was aseptically removed and confluent monolayer was washed with 2 ml of phosphate buffered saline (PBS). Then 1 ml of trypsin was added to detach the cells from the surface of the T-flask followed by incubation in humidified CO_2 incubator (37°C and 5% CO_2) for 2-3 minutes. After cells have detached (by examination under microscope) 4 ml of fresh medium was added to keep the cells in suspension and was homogenized by gentle flushing. Finally 1 ml of the cell suspension were reseeded into new T-flask containing 4 ml of fresh medium supplemented with 10% FBS.

3.4.5 Microcarrier Preparation for Culture

UVO treated PS microcarriers were sterilized by a series of washings using ethanol. First, microcarriers were soaked in Ca2+, Mg2+-free PBS to remove any contaminants. After microcarriers had settled, the supernatant was decanted and replaced with 70% (v/v) ethanol in distilled water. Microcarriers were washed twice with 70% (v/v) ethanol and incubated overnight in the same solution at concentrations of 50–100 mL g−1 microcarriers. The ethanol solution was then removed, and microcarriers were rinsed in sterile Ca2+, Mg2+-free PBS (50 mL g−1 microcarriers) twice, and once in the culture medium (20–50 mL g−1 microspheres). Microcarriers were re-suspended in the culture medium before use.

3.4.6 Cell Growth in Bioreactor Systems

3.4.6.1 T-flask Culture

The method for growing cells in 25 cm^2 t-flask is as described in the previous section (refer to Section 3.3.3). T-flask was inoculated with 1 ml of cell inoculum at a concentration of 1 x 10^5 cells/ml. Sampling was done every 12 hours to determine the cell growth.

3.4.6.2 Spinner Vessel

Cells were grown using a 500 ml spinner vessel (Medigene) with 200 ml working volume. The inner surface of the spinner vessel was coated with 5% silicon oil in ethyl acetate (to prevent microcarriers from attaching to the inner surface) beforehand. After sterilized by autoclaving, vessel was taken into the biosafety cabinet and 150 ml of culture medium supplemented with desired concentration of FBS was transferred aseptically into the vessel. It was then followed by inoculation of microcarrier suspended in 30 ml of culture medium and cells at a concentration of 10 x 10^5 cells/ml suspended in 20 ml of culture medium supplemented with the desired concentration of FBS. Spinner vessel was later transferred into the humidified CO_2 incubator and agitation was started by adjusting the agitation rate of the magnetic stirrer. Sampling was done every 8 hours to determine the cell growth.

3.4.7 Cell Counting in Different Bioreactors

For propagating viruses in cell culture, it is important to know the growth profile of the selected host cell line because infection need to be done at certain period during the cell's exponential growth phase. Therefore cell counting was performed at regular intervals to provide data to build the growth profile.

3.4.7.1 T-flask Culture

The trypan blue exclusion dye method described by Freshney (2005) was adopted. After cell trypsinization, as described in the previous section (refer to Section 3.3.3), cell suspension was mixed thoroughly by pipetting gently to disperse any clumps. Next 10µl of the cell suspension was transferred on a piece of glass slide and was mixed with an equal volume of trypan blue dye. Ten microlitres of the mixture was placed on the haemocytometer and allowed to spread by capillary action. Cells were scored live when they appear shining, while dead cells absorb the dye and appear dark blue. Only cells that were present in $1mm^2$ area were counted. Cell concentration (live and dead) was determined using the following equation:

$$c = \frac{n}{v} \qquad (3.1)$$

where c is the cell concentration/ml; n is the number of cells counted and v is the volume of sample counted. The area of squares of a haemacytometer slide is illustrated in Figure 3.3.

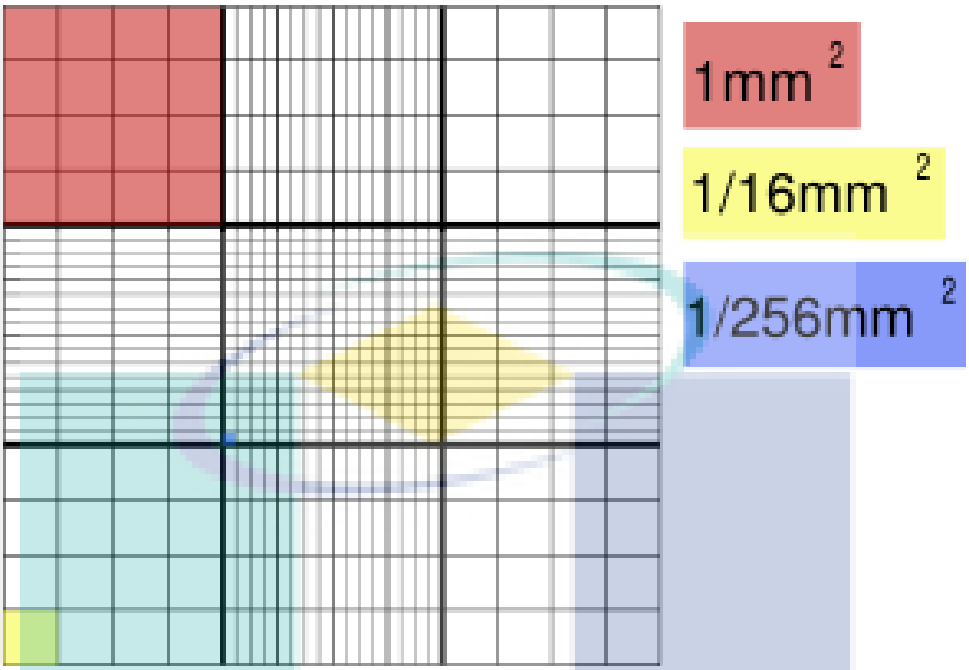

Figure 3.3: Counting grid of haemacytometer.

3.4.7.2 Spinner Vessel Culture

One mililiter of sample was placed in a test tube and after the microcarriers had settled, the supernatant was removed and the microcarriers were briefly washed in 2 ml of warm Ca^{2+}, Mg^{2+}-free PBS containing 0.02% (w/v) EDTA, pH 7.6. When the microcarriers had settled this solution was decanted and replaced by 1 ml of a1:1 mixture of 0.25% (w/v) trypsin in Ca^{2+}, Mg^{2+}-free PBS and EDTA (0.02%, w/v) in Ca^{2+}, Mg^{2+}-free PBS. The pH of this mixture should be 7.6. The tube was then incubated at 37°C for 15 minutes with occasional agitation.

After microcarriers had settled, the supernatant was transferred into another test tube. The microcarriers were washed with 2 ml of culture medium containing serum (5-10%, v/v) and the supernatant was pooled with the first supernatant. The cell suspension was centrifuged at 300 g for 10 minutes at 4 °C. The supernatant was discarded, and the pellet was resuspended in 2 ml Ca^{2+}, Mg^{2+}-free PBS containing 0.05% (w/v) trypan blue. The concentration of cells in the suspension

was counted using the same procedures used for T-flask. Including trypan blue in the resuspending solution allows estimates of cell viability to be made at the same time.

3.4.8 Specific Growth Rate and Doubling Time

Calculation of specific growth rate (μ_x) using the formula of Scheirer and Merten, (1991) was used:

$$\mu x = \left(\frac{\ln X_n - \ln X_{n-1}}{t_n - t_{n-1}}\right) \quad (3.2)$$

where X represents the viable cell density per ml, t the time points of sampling expressed in hours; n and $n-1$ stand for two succeeding sampling points. Doubling time (t_d) is given by the formula:

$$t_d = \left(\frac{\ln 2}{\mu_x}\right) \quad (3.3)$$

3.4.9 Cell Infection with NDV in Different Bioreactors

3.4.9.1 T-flask

Confluent monolayers of cells were used for infection with NDV as described by Hussain and Rasool (2005). Spent medium was removed and cell monolayer was washed with 5 ml of warm PBS. Then the cells were infected by 100 µl of NDV having virus titre of 128 HA and for lentogenic strains of NDV it was followed by addition of 100 µl trypsin. The virus inoculum was spread uniformly and incubated in humidified CO_2 incubator for 1 hour with intermittent rotation to allow adsorption. Five milliliters of maintenance medium with 2% FBS was added to flask. The flask was later returned to humidified CO_2 incubator and monolayers were examined daily under inverted microscope for evidence of CPE.

3.4.9.1.1 Harvesting of Virus

The virus was harvested by combination of freeze thawing, sonication and centrifugation process. The infected flasks were transferred to -80°C for 1 hour at 120 hours post inoculation and then thawed at room temperature. This process was later resumed with sonication at 15°C for 1.5 minutes. The virus suspensions were later poured into centrifuge tubes and centrifuged at 300g for 10 minutes at 4°C to pellet the cell debris. The clear supernatant fluid containing virus was collected carefully, divided into two aliquots, labeled as passage 1 (P1) and stored at -80 °C until further use.

3.4.9.1.2 Adaptation of Virus

The passaged virus (P1) was inoculated again to fresh monolayer of cells using the same technique and observed for CPEs (refer Section 3.4.9.1 and 3.4.9.1.1). The harvested virus sample was labeled as passage 2 (P2). Similarly passage 3 (P3) was obtained through the 3rd infection and CPEs were observed daily up to 120 hours post inoculation. The following passages were obtained using similar steps mentioned earlier.

3.4.10 Virus Analysis

Virus analysis was performed on virus samples produced in all bioreactor systems. In this study, haemagglutination assay (HA) was used to quantify the number of virus particles produced.

3.4.10.1 Haemagglutination Assay

The haemagglutination assay (HA) method described by (Grimes, 2002) was used. Using a 96 well V-shaped microtiter plate, 50µl PBS was added from wells 2A-12A. One hundred microlitres of virus sample was added to well 1A to which 50µl of it was serially diluted up to well 11A. Fifty

microlitres of 1% red blood cell (RBC) was added to all wells. Agglutination of RBC was observed in wells containing the virus while clumping of RBC was seen in wells containing no virus. Dilution of the last well showing agglutination gave the titre of the virus sample.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 INTRODUCTION

This chapter discussed the results for each study in detail started with the adaptation of Vero cells in Virus Production Serum Free Medium to ND virus propagation in adapted Vero cells. The production of UVO treated PS microcarriers and characterization are also discussed in this chapter.

4.2 ADAPTATION OF VERO CELLS IN VIRUS PRODUCTION SERUM FREE MEDIUM

Vero cells were grown in T-flask in 100% SSM, 25% SFM, 50% SFM, 75% SFM, and 100% SFM. Figure 4.1 below shows the detached cells from the wall of T-flask after trypsin is added and incubated for 5 to 10 minutes. The cells are observed through inverted microscope under 10X magnification. Figure 4.2 shows the cells at 12th hour under 20X microscope. Figure 4.3 shows the cells at 120th hour under 20X magnification.

From Figure 4.1, the shape of the cell is round after the cell detached from the wall of the T-flask. The cell must be detached during cell counting. However, when the cell is still attached to the wall of the T-flask, the cell is cylindrical in shape as shown in Figure 4.2 and Figure 4.3. Cells at 120th hour were more compact than 12th hour and all the surfaces on the wall of T-flask had been occupied by the cells. Cells in 120th hour were seen overlapping with each other.

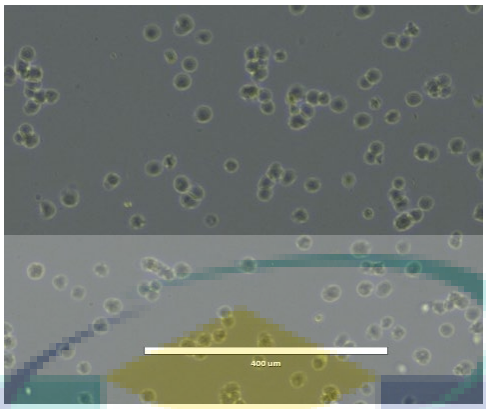

Figure 4.1: Detached cells at 10X magnification.

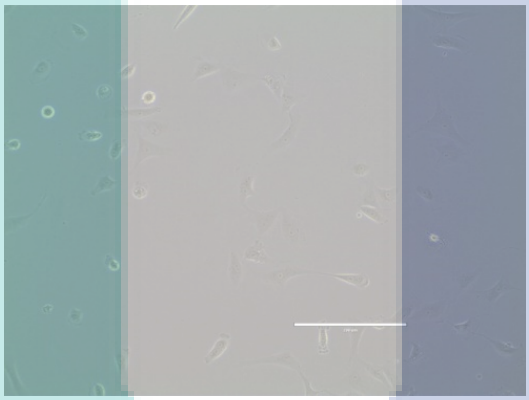

Figure 4.2: Cells at 12th hour under 20X magnification.

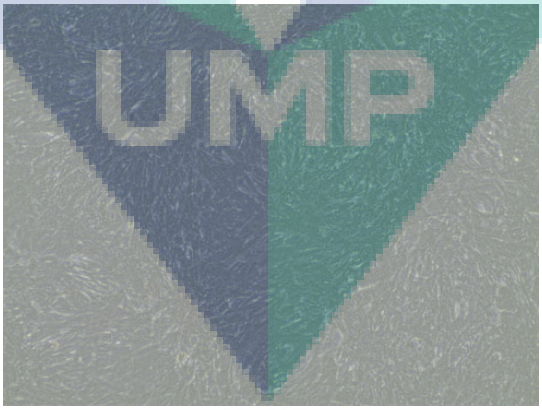

Figure 4.3: Cells at 120th hour under 20X magnification.

After the cells are grown in 100% SSM, the cells are adapted in serum free medium (SFM). The cells are adapted in SFM with increase concentration of SFM which are 25% SFM, 50% SFM, 75% SFM, and 100% SFM. The cells had to be counted for every 12 hours. Figure 4.4 below shows the graph of Vero cells that are grown in 100% SSM, 25% SFM, 50% SFM, 75% SFM, and 100% SFM.

As observed in Figure 4, the cells started with lag phase. The number of cells did not grow in number but grow in size (Harrison et al., 1997). This was because the cells started to get used to the new environment after sub-cultured from another flask. After lag phase, the cells entered exponential phase. The number of cells increased rapidly. This was because the cells duplicated themselves (Sinha et al., 2008). It had enough space, nutrient, and gases for it to grow and divide. They used the nutrients and excrete metabolites. After exponential phase, the cells entered death phase. The number of cells decreased and most of the cells died. This was because the concentration of the nutrients in the medium was decreasing rapidly. Besides, end-product that can hinder the growth of the cells such as lactic acid can cause death to the cells (Sinha et al., 2008). The space also had been limited where the anchorage-dependent cells were not able to attach to the wall of the flask. This caused the cells to overlap each other and multilayer of cells was produced. The concentration of oxygen in the medium was also not enough (Graff & McCarty, 1957).

As evidenced by Figure 4.4, culture in 100% SSM had the lowest cell maximum concentration compared to the cells that were adapted in SFM. The concentration of cell increased as the concentration of SFM increased.

Doubling time (Td) is used for quantification of Vero cells growth rate. It is usually determined from two volume estimation with measured time intervals comparable (Mehrara et al.,

2007). Table 4.1 below shows the maximum number of cells and their doubling time in each concentration of medium.

Table 4.1: The maximum cell concentration (cells/mL) and doubling time (hr) of cells at different concentration of medium.

Concentration of medium	Maximum cell concentration (cells/mL)	Doubling Time, T_d(hr)
100% SSM	1,062,500	35.20
25% SFM	1,400,000	37.82
50% SFM	1,412,500	37.69
75% SFM	1,515,000	36.72
100% SFM	1,595,000	33.83

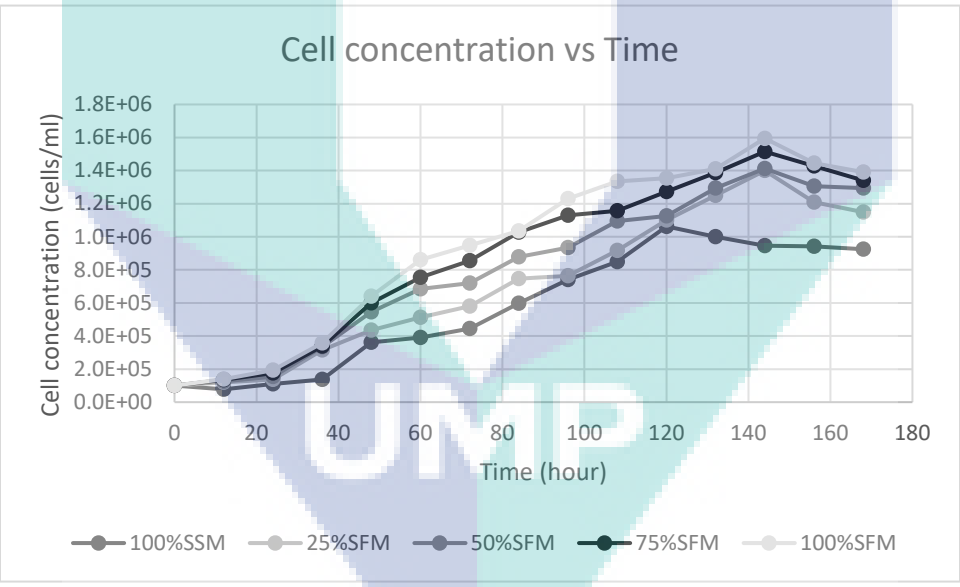

Figure 4.4: The graph of concentration of cells (cells/mL) against time (hour).

From Table 4.1, the cells that were grown in 100% SFM had shorter doubling time which is 33.83 hours compared to 100% SSM which is 35.20 hours. This means that it required shorter

time for the cell to reach maximum cell concentration when in 100% SFM compared to 100% SSM.

There are a few disadvantages of SSM which include batch-to-batch variation in composition, high protein content which prevent product purification, and has potential for viral, mycoplasma or prion contamination. As evidenced by results in this study, cells has better growth in SFM. SFM does not contain any animal products and has low protein content which is less than 90µg/L. In SFM, it contains recombinant growth factors and other non-proteinaceous cofactors. Lastly, it contained a range of trace elements which are important for long-term growth and stability of cells (Butler et al., 2000).

For the cells that were grown in 100% SSM, the main energy source was glucose. The concentration of glucose depleted along with time (Graff & McCarty, 1957) (Westfall et al., 1953) (Himmelfarb et al., 1969). The cells required glucose for proliferation and maintenance (Graff & McCarty, 1957). Besides glucose, cells also required other carbohydrates for synthesis of nucleic acid but not as source of energy (Griffiths, 1972) (Zielke et al., 1984) (McGowan et al., 1984). In SFM, it contained glutamine. The cells that were grown in SFM used glutamine for cell growth, survival, and maintenance.

During the cell growth, the cells used up all the nutrients. At the same time, the cells also excrete metabolic end products. The metabolic could either inhibit or stimulate the cell growth. The metabolic that could inhibit the growth of the cells are lactate, ammonia, and glutamate. Increase in the concentration of lactate can cause the pH of medium to decrease which inhibit the growth (Imamura et al., 1982). Ammonia can cause toxic to the cell if the concentration reached 2mM in medium (Thilly et al., 1982) (Holley et al., 1978). Beside metabolites, heat can be the source of the death of the cell.

Growth-stimulating endogenous metabolites are produced by the cells during the growth (Nahapetian et al., 1986). It promotes the growth of the cells. In 25% SFM, 50% SFM, and 75% SFM, they contain spend medium which consists of growth-stimulating endogenous metabolites. This promotes the cells to divide rapidly.

4.3 CHARACTERIZATION OF MICROCARRIERS

4.3.1 Fourier Transform Infrared Spectrum (FTIR) analysis

Figure 4.5 shows the IR spectra of PS microspheres before and after UVO treatment. By using OMNIC (Thermo Scientific, USA), a subtraction plot between spectra of untreated and UVO treated PS microspheres was obtained and is presented in Figure 4.6. This subtraction plot allows the software to identify new absorption bands and assign possible functional groups to each band. After a treatment time of 60 minutes, new absorption band appeared at 3421.31 cm^{-1} which marks the presence of O-H while an intense sharp band at 1732.82 cm^{-1} corresponds to C=O stretching of carboxylic acid and aliphatic esters (Murakami et al., 2203)(Davidson et al., 2005)(Yan et al., 2010). The treatment also has resulted in the appearance of a broad absorption band at 3300-2500 cm^{-1} which can be ascribed to O-H stretching of hydroxyls (Mitchell et al., 2005).

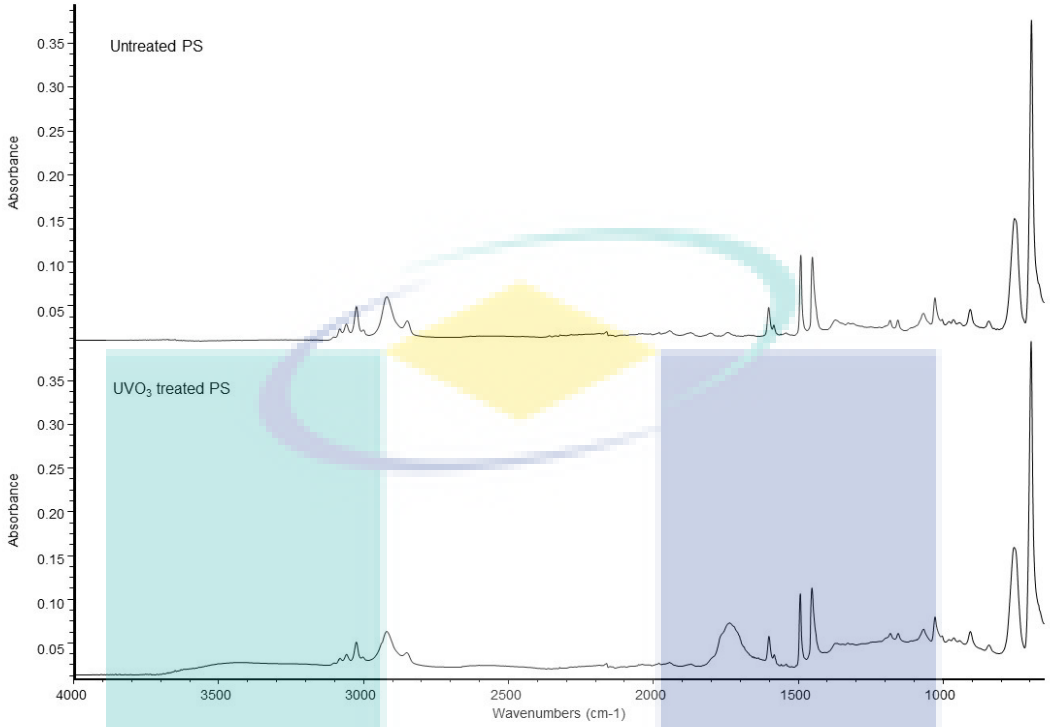

Figure 4.5: ATR/IR spectra of untreated and UVO treated PS microspheres

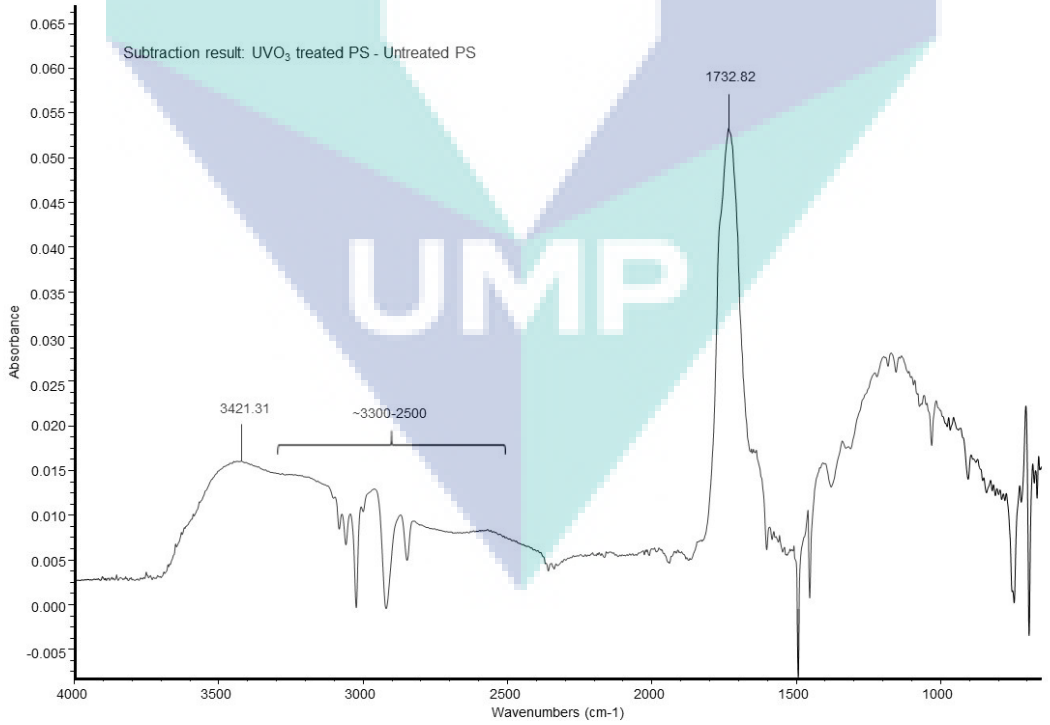

Figure 4.6: Subtraction result of ATR/IR plot; UVO treated PS *minus* untreated PS microspheres

4.3.2 Surface Carboxyl Quantification

As described in the FTIR analysis, treatment of PS microspheres with UVO system has successfully introduced carboxyl functional groups on the surface of microspheres. The amount of surface carboxyl measured by toluidine blue O (TBO) assay was 1501.61 nmol/g.

Carboxyl and hydroxyl are among functional groups that are potentially responsible for enhancing cell adhesion by improving surface wettability and increasing surface energy (Van Kooten et al., 2004) (Vogler and Bussian, 1987). Carboxyl enriched surfaces have been shown to promote adhesion of many cell lines including murine fibroblasts (Brandley et al., 1986), keratinocytes (Mitchell and Smith, 2012), LNCap cells (Sasai et al., 2008), and endothelial cells (Bhattacharyya et al., 2010).

4.3.3 Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDX)

Figure 4.7 shows the SEM image of untreated PS microspheres prepared by emulsion solvent-evaporation method at 0.25% (w/v) PVA, agitation speed of 300 rpm, PS to $CHCl_3$ (w/v) ratio of 1:5, oil to water phase (v/v) ratio of 1:5 and agitation temperature of 80°C. While Figure 4.8 shows the image of PS microspheres that has been treated by ozone at 77,728.24 ppm and irradiated by UV lamps at 0.343 mW/cm^2 for 60 minutes. As shown in both figures, all microspheres produced were perfectly spherical and had relatively smooth and non-porous surface. Smooth surface of microspheres indicates complete removal of solvents during the fabrication process (Dhanaraju et al., 2010). However, there were several UVO treated PS microspheres that have some residues attached to their surfaces (indicated by white arrow in Figure 4.8A and magnified in Figure 4.8B). These residues were believed to have originated from improper preparation procedures prior to SEM imaging. Nevertheless, EDX analysis on those residues revealed no contamination by elements other than carbon and oxygen (data not shown).

Table 4.2 shows the measurement of carbon and oxygen elemental and atomic content of PS microspheres before and after UVO treatment by EDX analysis. The elemental and atomic percentage of carbon on PS microspheres before UVO treatment was very high, at 95.48% and 96.57% respectively. After the treatment, both carbon elemental and atomic content dropped significantly to 78.20% and 82.69% while

oxygen content increased sharply to 21.80% (element) and 17.31% (atomic). This result is in agreement with FTIR analysis and TBO assay described earlier in which UVO treatment has successfully introduced oxygen species such as hydroxyl, carbonyl and carboxyl functional groups onto the surface of PS microspheres.

Table 4.2: EDX data showing carbon and oxygen content on untreated and UVO treated PS microspheres

Sample	Element	Element (%)	Atomic (%)
Untreated PS	Carbon	95.48	96.57
	Oxygen	4.52	3.43
UVO PS	Carbon	78.20	82.69
	Oxygen	21.80	17.31

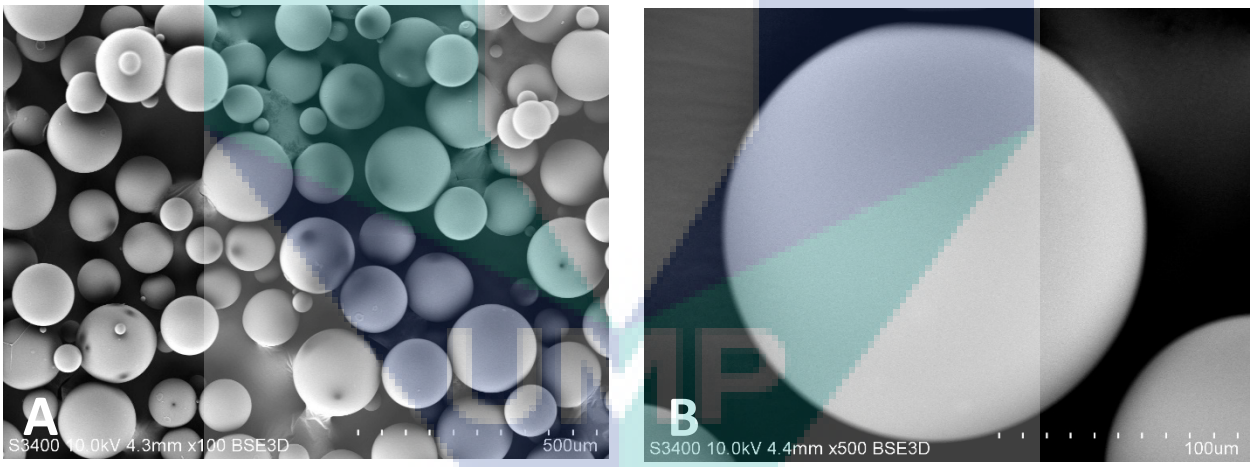

Figure 4.7: SEM photographs of untreated PS microspheres at different

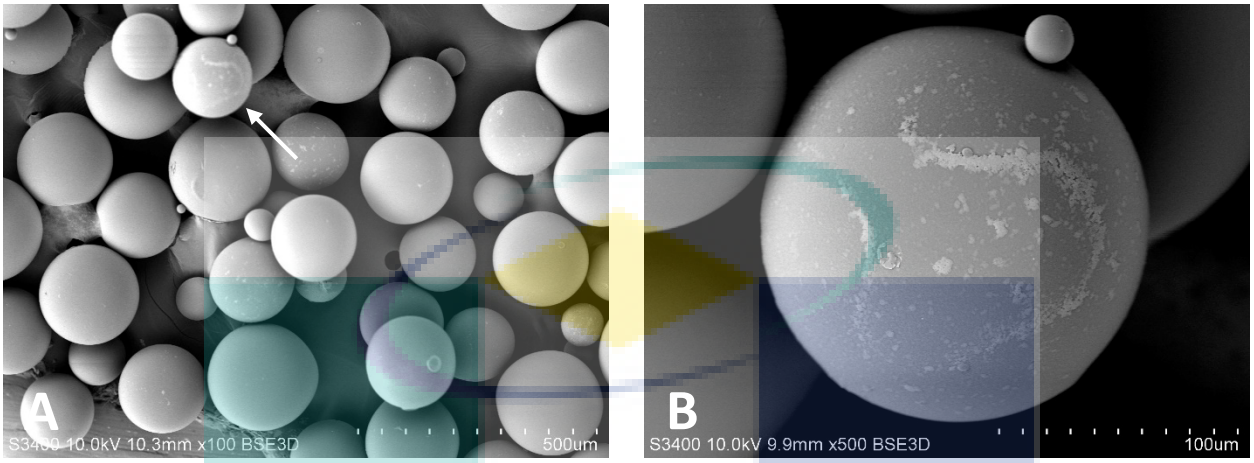

Figure 4.8: SEM photographs of UVO treated PS microspheres at different magnifications

4.4 CELL GROWTH ON MICROCARRIERS

The growth of Vero cells on UVO treated PS microcarriers achieved maximum cell concentration of 2.07×10^6 cells/ml 60 hours after cell inoculation (Figure 4.9). This marks fivefold increase in cell concentration when compared to cell inoculum size of 2×10^5 cells/ml. For untreated PS microcarriers, Vero cell concentration was low throughout the whole culture time. Cell concentration dropped significantly to 7.5×10^3 cells/ml 2 hours after cell inoculation. Following that, cell concentration fluctuated between 5×10^3 cells/ml and 2.88×10^5 cells/ml until the end of culture time.

Images of Vero cell growth on UVO treated, and untreated PS microcarriers are presented in Figure 4.10 and 4.11. As observed in Figure 4.10A, many cells have adhered and proliferated on microcarriers as early as 2 hours after inoculation. After occupying the whole surface area of microcarriers, Vero cells continued to multiply by forming cell multilayers on the surface of microcarriers and later formed microcarrier agglomerates in which more cells were proliferated in the cavity area of the agglomerates. After reaching maximum cell concentration at 60 hours (Figure 4.10B), cell multilayers on the agglomerates started to thin resulting from cell death. On the other hand, Vero cells did not grow well on the surface of untreated PS microcarriers because the hydrophobic surface provided does not promote cell adhesion and

proliferation (Figure 4.11A). Survived cells formed clumps and grew freely in suspension as seen in Figure 4.11B.

To further evaluate the growth of Vero cells on UVO treated PS microcarriers, several SEM images during culture were taken. As observed in Figure 4.12A and B, almost all microcarriers are coated with thick layers of Vero cells. Adhesion of cells to cells formed bridges between microcarriers, which led to microcarrier agglomeration during culture.

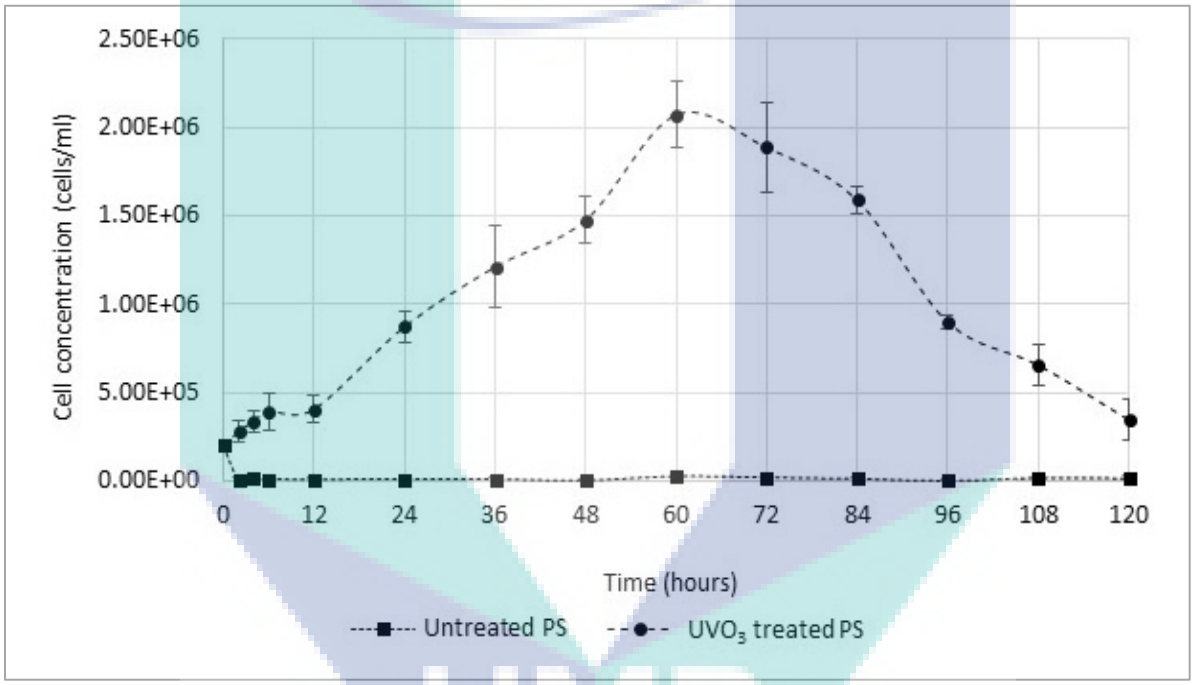

Figure 4.9: Growth profile of Vero cells grown on untreated PS and UVO treated PS microcarriers

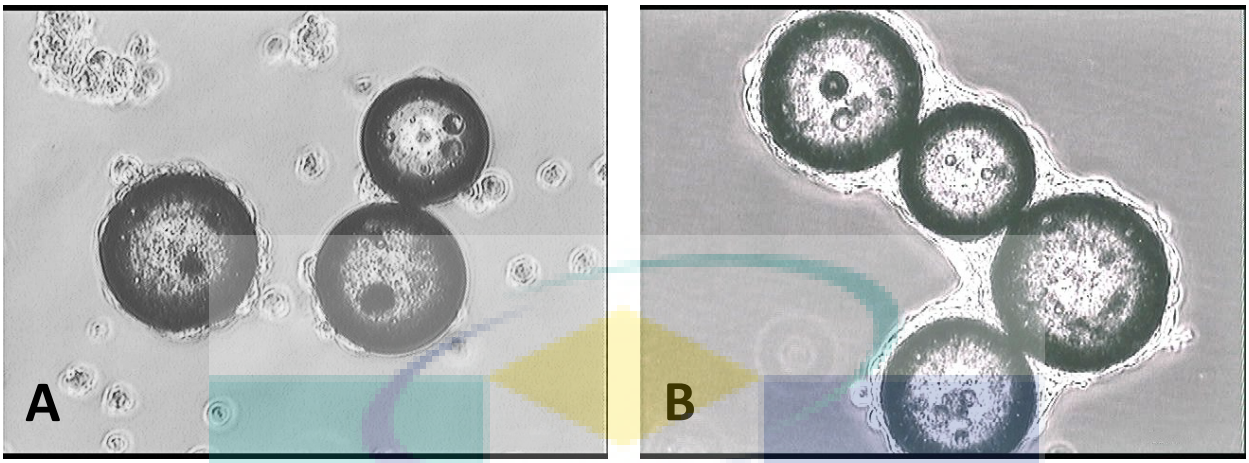

Figure 4.10: Vero cell attachment on UVO treated PS microcarriers viewed under inverted light microscope at different times of culture: a) 2 hours and b) 60 hours

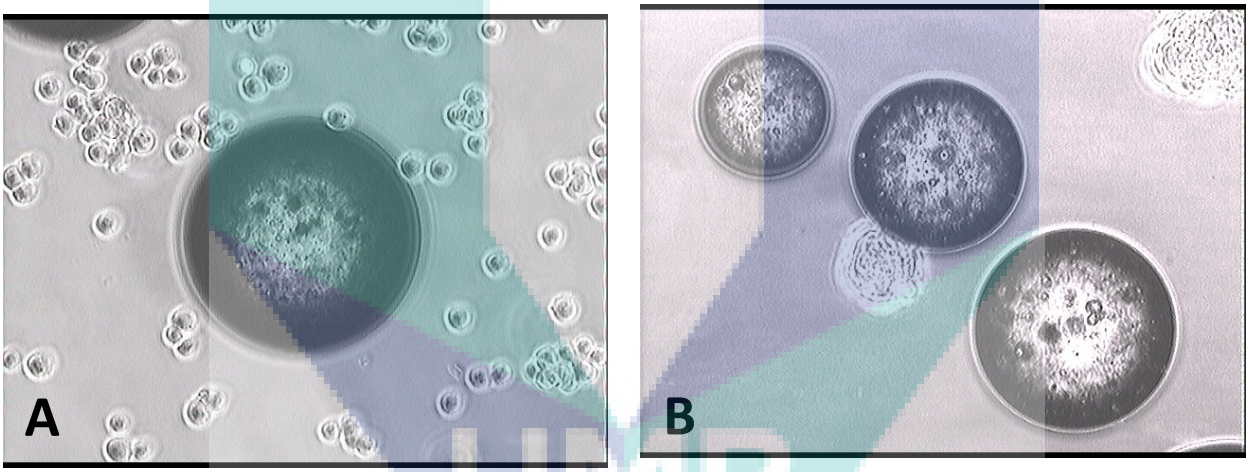

Figure 4.11: Vero cell attachment on untreated PS microcarriers viewed under inverted light microscope at different times of culture: a) 0 hours and b) 60 hours

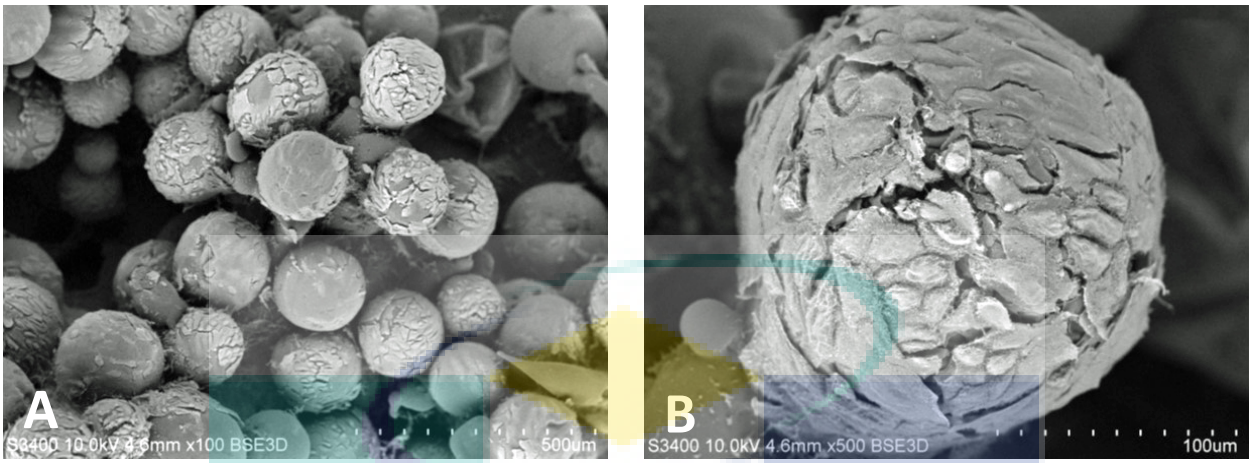

Figure 4.12: SEM photographs of Vero cell attachment on UVO treated PS microcarriers at different magnifications

4.5 NEWCASTLE DISEASE VIRUS (LA SOTA STRAIN) ADAPTATION

Adapted Vero cells were infected with mesogenic La Sota strain of NDV having initial HA titer of 128 HA units during their exponential phase. For all five passages, all samples failed to produce visible CPE in the culture and hemagglutination in the HA test. Passaging of normal looking cell cultures or blind passage was continued to encourage the appearance of visible CPE. The amount of virus produced in the first and second passages was assumed to be insufficient to produce those effects (Cann and Irving, 1999). However, for the following three passages, there was still no signs of CPE and no hemagglutination, therefore, passaging of NDV in adapted Vero cells was stopped.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

The experiments were performed to establish a model for producing Newcastle disease virus (La Sota strain) using UVO treated PS microcarriers. Numerous techniques and literatures concerning the preparation on cell and virus, polymer microspheres and polymer surface modification using UVO treatment have been successfully adapted. Based on all study components that have been discussed in the earlier chapters, the following conclusions are drawn:

i. The selected host cell line, Vero cells, have been successfully adapted in Virus Production Serum Free Medium (VP-SFM).
ii. UVO treated PS microcarriers have been successfully prepared and characterized.
iii. Propagation of La Sota strain of ND virus in the adapted Vero cell line were unsuccessful.

5.2 RECOMMENDATIONS

Based on the results obtained and observations throughout this research study, the following recommendations are proposed:

i. The potential of UVO treated PS microcarriers in high density culture of animal cells can be further improved by introducing porous structures.
ii. Different strategies or different host cells must be employed for the propagation of ND virus in cell culture.
iii. Different and stronger vaccine strains of ND virus may be employed in the suspension culture using UVO treated PS microcarriers.

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APPENDIX

List of publications

i. Surface modification of PCL microcarrier for performance improvement of human skin fibroblast culture. IOP Conf. Series 2018

ii. Optimization of Ultraviolet/Ozone (UVO) Process Conditions for The Preparation of Gelatin Coated Polystyrene (Ps) Microcarriers, submitted to 5th International Conference of Chemical Engineering & Industrial Biotechnology and Chemical Engineering and Processing: Process Intensification journal