

Virus-Based Nanoparticles for Tumor Selective Targeting and Oncolysis

By

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Abstract

Many oncolytic virotherapies have shown great advantages for rapid, rational design through recombinant DNA technology to facilitate the targeting of a broad spectrum of malignancies. Newcastle disease virus (NDV), an avian paramyxovirus, is naturally tumor-selective and inherently oncolytic. Our approach is to develop NDV-based nanoparticles (VBNP) for oncolytic virotherapy. VBNPs are non-infectious and non-replicating and are relatively safe. We obtained VBNPs by co-expressing matrix (M), hemagglutinin (HN), and fusion (F) proteins of NDV in avian/ mammalian cells. The budding characteristics, size and morphology of VBNPs were similar to authentic virions. As a proof of concept, we engineered the apoptin (VP3) gene of chicken anemia virus in VBNPs and specifically targeted them to folate-receptor bearing tumor cells by surface conjugation to folate. The VBNPs killed tumor cells by apoptosis and induced proinflammatory and chemotactic cytokines. The VBNPs, although not curative, were able to limit the progression of xenotransplanted fibrosarcoma

and malignant glioma tumors and provided a survival advantage in nude mice. We also engineered NDV M based particles with nipah virus surface glycoproteins to target ephrin B receptors. NDV based nipah Virus BNPs (NiV-ndBNP) were morphologically similar to authentic NiV virions. NiV glycoproteins were incorporated into the NDV M based particles, despite poor sequence homology in the transmembrane domain and cytoplasmic tails of glycoproteins. Our results suggest that VBNPs could be used to deliver small molecules, tumor antigens, anti-tumor/ reporter genes and also aid in generating tumor specific immunity by rational design.

Dedication

I would like to dedicate my thesis to my family and friends. To my parents, Mr. Pramod Chavan and Mrs. Shalaka Chavan for their love, support and encouragement, and to my pet dog, Sophie, her presence in my life pushed me to do my best for animals. This work is a result of the trust and faith they always had in me.

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Attribution

Several colleagues and coworkers aided in the writing and research behind several of the chapters of this thesis. A brief description of their background and their contributions are included here.

Chapter 3: Virus-Based Nanoparticles for Tumor Selective Targeting and Oncolysis

Dr. Elankumaran Subbiah- Ph.D. (Department of Biomedical Sciences and Pathobiology, Virginia Tech) is the Major Advisor and Committee Chair. Dr. Subbiah and I conceived, designed the experiments, performed statistical analysis, data analysis and processing and wrote the paper.

Shobana Raghunath - Ph.D. student (Department of Biomedical and Pathobiology, Virginia Tech) in Dr. Subbiah's Lab and contributed reagents, materials and analysis tools.

Adria Allen- Ph.D. student (Department of Biomedical and Pathobiology, Virginia Tech) in Dr. Subbiah's Lab. Adria contributed towards my research by helping with experiments.

Moanaro Biswas - Ph.D. (Department of Biomedical and Pathobiology, Virginia Tech) post-doctoral fellow in Dr. Subbiah's Lab. Moanaro helped me perform experiments.

Abilash P.S - Ph.D. student (Department of Biomedical and Pathobiology, Virginia Tech) in Dr. Subbiah's lab. Abilash contributed in performing some of the experiments.

Nikorn Pothayee - Ph.D. student (Department of Chemistry, Virginia Tech) in Dr. Judy Riffle's lab and contributed by providing reagents and materials for the experiments.

Dr. Judy Riffle - Ph.D. (Professor, Department of Chemistry, Virginia Tech) collaborated with Dr. Subbiah on this project and provided technical consultancy and reagents

Dr. Tanya LeRoith - Ph.D (Department of Biomedical and Pathobiology, Virginia Tech) is a

professor of Anatomic Pathology and she analyzed histopathology sections for the experiments.

Chapter 4: Surface Modification of Newcastle disease Virus-Based Nanoparticles with Nipah Virus Glycoproteins

Dr. Elankumaran Subbiah- Ph.D. (Department of Biomedical Sciences and Pathobiology, Virginia Tech) is the Major Advisor and Committee Chair. Dr. Subbiah and I conceived, designed the experiments, performed statistical analysis, data analysis and processing and wrote the paper.

Moanaro Biswas - Ph.D. (Department of Biomedical and Pathobiology, Virginia Tech) post-doctoral fellow in Dr. Subbiah's Lab. Moanaro assisted with some experiments.

Abilash P.S - Ph.D. student (Department of Biomedical and Pathobiology, Virginia Tech) in Dr. Subbiah's lab. Abilash contributed reagents, materials and analysis tools.

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List of Abbreviations

ACS	American Cancer society
CNS	Central Nervous System
CT	Cytoplasmic tail
CTL	Cytotoxic T Lymphocytes
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
EtBr	Ethidium Bromide
FCS	Fetal calf serum
Fo	Folate
FR	Folate Receptor
FA	Folic Acid
F	Fusion
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GPI	Glycosylphosphatidylinositol
HN	Hemagglutinin- neuraminidase
H/E	Hematoxylin and Eosin
HeV	Hepatitis E virus
HSV-1	Herpes Simplex Virus 1
IF	Immunofluorescence
IFN	Interferon
L	Large polymerase
MHC-1	Major Histocompatibility type I
M	Matrix

MMP	Matrix metallo-proteases
MeV	Measles virus
miRNA	MicroRNA
MuV	Mumps virus
NHS	N-hydroxysuccinimide ester
NDV	Newcastle disease Virus
NVNDV	Neurotropic velogenic NDV
NiV	Nipah Virus
NP	Nucleoprotein
Nt	Nucleotide
OV	Oncolytic Virus
OVT	Oncolytic virotherapy
PIV	Parainfluenza virus
PNS	Peripheral nervous system
PPRV	Peste des Petits ruminants virus
PBS	Phosphate Buffered Saline
P	Phosphoprotein
PTA	Phosphotunstic acid
PEG	Polyethylene glycol
PNP	Purine Nucleoside Phosphorylase
Rb	Retinoblastoma
RSV	Respiratory syncytial virus
RNA	Ribonucleic acid
RNP	Ribonucleoprotein core
RPV	Rinderpest virus
SDS-PAGE	Sodium-dodecyl sulphate polyacrylamide gel electrophoresis

TEM	Transmission Electron Microscopy
TM	Transmembrane
UTR	Untranslated Region
VBNP	Virus based nanoparticles
VLP	Virus like particles
VP3	Apoptin protein
VVNDV	Viscerotropic velogenic NDV
VSV	Vesicular Stomatitis Virus

Chapter 1: Introduction

1.1 Background

Cancer is a disease wherein a cell, or a group of cells exhibit uncontrolled growth, invasion and sometimes metastasis. These properties of cancer differentiate it from benign tumors, which are self-limited, and do not invade or metastasize. Cancer affects people at all ages but the risk of cancer increases with age (Cancer Research, January 2007). It is responsible for causing the highest number of human deaths every year. Cancer resulted in about 13% of all human deaths in 2007 (American Cancer Society, 2007; WHO, February 2006). Many factors contribute towards the etiology of cancer in humans. About 90-95% of cancers are caused due to lifestyle and environmental factors like diet and obesity, infections, tobacco, radiation, stress, lack of physical activity, environmental pollutants, which cause abnormality and mutations in the genetic material of cells (Anand et al., 2008; Kinzler and Vogelstein, 2002).

Over the past decade, several attempts have been made toward understanding cancer biology and discoveries emerging from cancer genomics that can be made beneficial for cancer patients along with radiation and chemotherapy as standard of care (Ladanyi and Hogendoorn, 2011). In view of the deleterious effects of cancer therapy with radiation and chemicals, alternative treatments like the use of viruses for cancer therapy is becoming popular. Certain viruses have tumor killing (oncolytic) property and this channels a new pathway for cancer therapeutics (Kelly and Russell, 2007). Gene therapy is another technology which aims at the correction of inherited genetic diseases by providing to the targeted cells a functional copy of the deficient gene responsible for the disease, in particular cancers (Touchefeu et al., 2010).

1.2 Justification

Oncolytic virotherapy (OVT) is the deliberate use of lytic viruses to infect and kill cancer cells (Adrienne et al., 2010). The field of OVT gradually developed through the use of promising animal tumor models. It was reported that both vaccinia virus and herpes virus could be propagated in mouse and rat skin tumors and sarcomas (Levaditi and Nicolau, 1922; Moore, 1952). This gave a hint in the use virus infection of cancer cells might show promise as a cancer therapy. More viruses were discovered and small animal tumor models were created over the next several years. There have been multiple clinical trials using OV's and in combination with immunosuppressive drugs like cyclophosphamide; retargeting of viruses to specific tumor types; using infected cell carriers to protect and deliver the virus to tumors; and genetic manipulation of the virus to increase viral spread and/or express transgenes during viral replication (Lech and Russell, 2010).

OV's are appealing as cancer therapeutics since they can be genetically manipulated and they exhibit multiple discrete anticancer mechanisms. OV's cause direct tumor cell killing, independent of conventional drug-resistance mechanisms (Coukos et al., 2000). Notably, they also have the ability for self-propagation, thereby effectively spreading throughout a tumor and being active against massive and potentially metastatic disease. Viruses can also be used to deliver therapeutic genes by arming the viruses, which further enhances the antitumor capabilities of these viruses (Hermiston, 2000), and can cause substantial modification in the tumor microenvironment (Kaur et al., 2009). They can possibly sensitize the host immune system to tumor-specific antigens that in other instances would not have been immunogenic, hence acting as an *in situ* cancer vaccine (Benencia et al., 2008; Toda et al., 1999). In the current epoch of molecular biology, OV's are a potentially useful therapy to reduce bulky disease, alone or in combination with conventional therapies, and as an immunotherapy to prevent cancer (Hammill

et al., 2010). However, in instances where the cancer patients are immunocompromised and the host antiviral immunity restricts OV replication, other OV-based options that would retain the inherent ability of OV to kill cancer cells and evoke antitumor immunity, are necessary. The use of non-replicating viruses or virus-like particles that could be engineered for oncolytic efficacy is an attractive strategy.

1.3 Hypothesis

We hypothesize that virus-based nanoparticles from a promising OV such as avian Newcastle disease virus (NDV) could be engineered for selective retargeting and arming for cancer therapy.

1.4 Objectives

1. To study the requirements for the formation of NDV-based nanoparticles
2. To characterize the budding and morphology of NDV-based nanoparticles in human cells
3. To determine whether NDV-based nanoparticles will be cytotoxic to cancer cells
4. To explore selective retargeting of NDV-based nanoparticles to folate receptors
5. To determine whether it would be possible to arm NDV-based nanoparticles with a model proapoptotic gene
6. To examine whether NDV-based nanoparticles will induce anti-tumor immune response in select cancer cell populations *in vitro*
7. To determine whether it would be possible to surface modify NDV-based nanoparticles with envelope glycoproteins from other paramyxoviruses for efficient targeting
8. To determine whether NDV-based nanoparticles can cure or prolong survival in xenotransplanted pre-clinical mouse models of fibrosarcoma and malignant glioma.

1.5 Thesis Outline

This thesis consists of five chapters. Chapter one introduces the research topic, while chapter two reviews current research being done in related areas. Subjects discussed include use of viruses for cancer therapy, Newcastle disease virus as an oncolytic virus, need for better OV platforms and methods for shielding, arming, and targeting oncolytic viruses or virus-based platforms. Chapter three presents the research completed to determine the efficacy of virus-based nanoparticles for cancer therapy. Additionally, this chapter will discuss the ability to engineer foreign antigens on NDV envelope and folate targeting for cancer therapy. Chapter four will present the results of surface modification of NDV-based nanoparticles with Nipah virus envelope glycoproteins and the options available for efficient targeting to select cell types using this approach. Finally, chapter 5 will summarize the results and provide conclusions and areas for future research..

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Chapter 2: Literature Review

2.1 Introduction

In the last few decades, many novel paramyxoviruses have emerged causing catastrophic diseases in different species of animals and some of them also made the species jump to humans. Members of the family *Paramyxoviridae* have been isolated from diverse host species and they are found to be associated with central nervous and respiratory system diseases (Lamb and Parks, 2007). There are many important viruses in this family, including animal pathogens that are economically important in poultry and livestock industry, such as Newcastle disease virus (NDV) (Alexander, 2009), Rinderpest virus (RPV) (Roeder and Taylor, 2002) and *Peste des Petits ruminants* virus (PPRV) and human pathogens such as measles virus (MeV), mumps virus (MuV), respiratory syncytial virus (RSV), and parainfluenza viruses (PIV) (Lamb and Parks, 2007). Some members of the *Paramyxoviridae* family such as MeV and NDV are also being extensively studied as a vaccine vector or for use as anticancer agents (Cassel and Garrett, 1965; Elankumaran et al., 2006; Russell and Peng, 2009).

Paramyxoviruses are enveloped RNA viruses possessing non-segmented, negative strand genomes in the order *Mononegavirales* (Loney et al., 2009). Family *Paramyxoviridae* can be divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*, based on virion morphology, genome organization, and structure and sequence relatedness. Currently, there are five genera within the subfamily *Paramyxovirinae*: Rubulavirus, Avulavirus, Respirovirus, Morbillivirus, and Henipavirus, and two genera within the subfamily *Pneumovirinae*: Pneumovirus and Metapneumovirus (Lamb and Parks, 2007). Respiroviruses and rubulaviruses demonstrate both hemagglutinating (HA) and neuraminidase (NA) activity. Morbilliviruses and henipaviruses exhibit only HA but lack NA activity. Pneumoviruses are morphologically differentiated by their narrower nucleocapsids from *Paramyxovirinae*. Pneumovirus does not cause substantial HA in

mammalian and avian erythrocytes (Lamb and Parks, 2007).

They have a close relationship with two other negative strand RNA viruses: Rhabdoviruses (for their unique non-segmented genome organization and its expression) and Orthomyxoviruses (for the biological properties of the envelope glycoproteins (Lamb and Parks, 2007)).

2.2 Newcastle Disease Virus

Newcastle disease virus (NDV) is responsible for causing a highly contagious disease in many species of domestic and wild birds. The disease was first reported in Java in 1926 and was brought to international attention in Newcastle on Tyne, England, in 1926 (Alexander, 2001; Shope, 1964). The disease is characterized by the presence of respiratory, digestive and neurological signs in many avian species. The severity of clinical signs ranges from an inapparent infection to a rapidly fatal condition (Alexander, 2001). Standard pathotyping assays utilize inoculation of embryonated eggs and live chickens to determine the virulence amongst different strains of NDV. NDV strains are classified as velogenic (highly virulent), mesogenic (moderately virulent) and lentogenic (avirulent) depending on the severity of the disease they cause (Alexander, 2000; Beard and Hanson, 1984). The velogenic strain is further divided into two pathotypes, the viscerotropic velogenic (VVNDV), which cause acute lethal disease with visceral hemorrhages and the neurotropic velogenic (NVNDV) responsible for neurological and respiratory signs.

NDV genome is about 15,186 nucleotides long and encodes 6 genes, placed 3'-NP-P-M-F-HN-L-5', with short untranslated regions (UTRs) at either end of each gene. The six NDV genes encode structural and non-structural proteins that are classified into membrane and core components. The membrane components consist of two transmembrane glycoproteins, the Fusion

(F) and hemagglutinin (HN) proteins, and the matrix (M) protein (Fig. 2.7). The virus envelope encases the ribonucleoprotein (RNP) core, which is formed by the RNA genome associated with the nucleocapsid protein (NP) and the polymerase complex composed of phosphoprotein (P) and large polymerase (L) proteins. The inner surface of the virion is lined by the M protein which probably mediates the interaction between the RNP complex and lipid-bilayer, as well as the cytoplasmic tails of the spike glycoproteins (Cathomen et al., 1998a). The structure of NDV is presented as a schematic cartoon in Fig. 2.7.

2.3 Oncolytic Viruses

Cancer is a major cause of deaths in humans. Though there has been significant progress in cancer therapy, the limited efficacy and toxicities of current chemo- and radiotherapies have provided an impetus for the search of novel therapeutics. A therapeutic approach, which uses viruses for the treatment of cancer termed, oncolytic virotherapy has recently emerged (Kelly and Russell, 2007). Oncolytic virus (OV) is a virus that preferentially infects and lyses cancer cells. OVs have achieved a high recognition in cancer therapy, both by directly destroying the tumor cells, and by having the capacity to be modified as vectors. They can carry genes that express anticancer proteins and deliver them specifically to the tumor site. Most current OVs are engineered for tumor selectivity, but there are a few naturally occurring OVs. The safety and efficacy of OVs were widely debated, and studied in both subjective and official clinical trials since 1950s. Early virotherapy clinical trials based on natural viruses were poorly directed, but recently, modified viruses have been subjected to extensive screening of viral replication, gene expression, and host immunity (Liu et al., 2007). Several strategies can be applied to many viruses to augment therapy (Cattaneo, 2010; Cattaneo et al., 2008).

Over the past few years, there have been new insights on the molecular mechanisms of viral cytotoxicity, which provided the scientific rationale to design more effective OVs. Several

recent clinical trials have used genetically engineered viral strains, such as adenovirus and herpes simplex virus 1 (HSV-1) as well as wild-type Newcastle disease virus, which show promising results with these viruses being relatively non-toxic and tumor specific. But how do OV's specifically target cancer cells, and what is the potential for using OV's as cancer therapeutics? Replicative selectivity of viral genes can be modified for proficient replication, so that the virus can only replicate in cells that have interruptions in normal homeostatic pathways, such as tumor-suppressor defects or activation of oncogenic pathways (Chiocca, 2002). OV's, along with their positive use in virotherapy come with a few disadvantages. Certain OV's such as the laboratory engineered HSV-1, adenovirus, can easily be manipulated genetically but induce side effects which include serious or potentially fatal disease in immunocompromised individuals. Also, the engineered OV's like reovirus and VSV are not easy to manipulate genetically. Most OV's are also limited by the host antiviral response.

Arming oncolytic viruses with transgenes that are capable of inducing apoptosis is a striking strategy for improving anti-cancer activity (Cattaneo et al., 2008). Three principles for developing more specific and potent OV's have been employed, for future clinical trials, namely: targeting, shielding, and arming (Cattaneo et al., 2008).

2.3.1.Targeting OV's:

During infection, viruses bind to one or more host cell surface proteins, and the viral tropism can be determined by tissue-specific expression of these proteins. A range of chemical and genetic engineering strategies have been tested to retarget the cell entry of both enveloped and non-enveloped viruses through specific cancer-cell-specific receptors (Waehler et al., 2007). Paramyxoviruses have contributed to the development of the next generation of cancer therapeutics and in particular on targeting viral entry to cancer cells. The paramyxovirus

envelopes can target substrate by receptor attachment and fusion, which functions on two separate proteins. In contrast, a single protein in retroviruses performs both functions, which has complicated retargeting strategies. The two-protein entry system of paramyxoviruses is also simpler than those of large DNA viruses that use several proteins. Among paramyxoviruses, targeting of the measles virus (MeV) envelope is the most advanced (Cattaneo, 2010).

OVs are cytotoxic and target cancers via multiple mechanisms of action and at the same time they utilize authenticated genetic pathways, which are dysregulated in nearly all cancers. In tumor cells, cell growth is unregulated, with many human cancers harboring mutations in p53 or Retinoblastoma (Rb). A vast majority of cancers arise as a result of the accumulation of multiple genetic mutations. But only in very specific cases, the detection and consequent targeting of tumors is feasible. Subsequently, a plethora of therapeutic approaches targeting the related pathways/key players that support or are crucial for tumor development are being developed and explored for effective targeting and virotherapy (Ferrara et al., 2004; Pavet et al., 2010; Tennant et al., 2010). Some tumors exhibit certain common characteristics that are at the basis of anticancer drug development (Bianco et al., 2007; Desgrosellier and Cheresch, 2010; Granchi et al., 2010; Tennant et al., 2010). Current approaches target three features of tumor growth, i.e. self-sufficiency in growth signals, sustained angiogenesis and resistance to apoptotic stimuli, and have been shown to be successful in some cases (Desgrosellier and Cheresch, 2010; Pavet et al., 2010; Tennant et al., 2010).

Virus reprogramming has been achieved with several targeting strategies that exploit either receptor-mediated entry or by particle activation (eg. via proteases), which was based on mutating the F-protein in paramyxoviruses that required protease cleavage for activation. Both Sendai virus and MeV were used for this targeting strategy (Cattaneo, 2010; Springfield et al.,

2006; Wolfe et al., 2004b). Also targeting the transcriptional and replication steps (eg. promoters, cancer cell defects) was found to be effective.

2.3.2 Shielding the OV's

Once the entry of the OV's is achieved, the body's immune mechanism kicks in to eliminate any foreign body from the system. In order to accomplish a successful effect of OV's after entry, a strategy to shield the OV's before it gets degraded by remodeling the viral or serotype envelope, coating a polymer by chemicals, biological cell carriers or simply by intratumoral/specific injections is successful in the recent years. These methods have been used to shield icosahedral viruses, in particular adenovirus. The envelopes of non-human paramyxoviruses can be modeled into MeV nucleocapsids to produce chimeric viruses that would help to evade the pre-existing MeV immunity for a short period of time till the desired effect has been achieved (Cattaneo et al., 2008).

2.3.3 Arming the OV's

Arming OV's with pro-apoptotic genes, pro-drug convertases like Purine Nucleoside Phosphorylase (PNP) or fludarabine, or selective disarming in normal cells like interferons, granulocyte-macrophage colony stimulating factor (GM-CSF) are being explored (Cattaneo, 2010; Cattaneo et al., 2008). Suppressing the host immune system with cyclophosphamide before virus administration may likely enhance the oncolytic efficacy, possibly by suppressing host innate and adaptive immunity and momentarily supporting virus replication. Arming of OV's enhances the efficacy of virotherapy by directly integrating it into a chemotherapy treatment, locally thereby amplifying its efficacy (Cattaneo, 2010).

2.3.4 Tumor Selectivity

A strategy of OV's to target replication of the attenuated virus is achieved by mutating viral functions that are necessary for replication in normal cells. This gives the virus the attenuated phenotype that leads to replication only in cells that are permissive such as dividing tumor cells or cells with defects in specific cancer pathway and sparing normal cells (Manservigi et al., 2010; Martuza et al., 1991; Meignier et al., 1990). Recent research highlighted another strategy to target viral replication by interfering the expression of essential viral genes that are controlled by tumor or tissue-specific promoters, which are preferentially active in tumor cells (Chung et al., 1999; MacLean et al., 1991; Mullen et al., 2002). Tumor cells with a defective or truncated Interferon (IFN) pathway escape the antitumor activity of IFNs and are selected (Vigil et al., 2007). Normal cells have an effective antiviral response and hence they are capable of inhibiting viral replication before a major damage is prompted. This characteristic defects of the IFN pathway on tumor cells elucidate the tumor-selective replication of some IFN-sensitive RNA viruses such as Reovirus, NDV and VSV, thereby providing a mechanism for using these OV's as safe and effective cancer therapeutic agents (Vigil et al., 2007).

2.4 Oncolytic Viruses in Clinical trials

In recent years, OV's have been genetically engineered to target malignant cancer cells selectively. Adenovirus is an OV, which is being tested in clinical trials for the treatment of cancer. The cells that are infected with replication-competent adenoviruses undergo autophagy, which has granted new opportunities for investigating the mechanism of adenovirus-induced cell death (Gomez-Manzano and Fueyo, 2010). Several studies have established that inserting microRNA (miRNA)-targeted sequences into the adenoviral genome can transform adenoviral protein expression for tissue and tumor selectivity (Gomez-Manzano and Fueyo, 2010). Many HSV-1 vectors have been developed and studied so far for specific gene therapy treatments

involving the central (CNS) and peripheral (PNS) nervous systems using different routes of inoculation in order to efficiently deliver genes into the CNS and PNS for treating neurodegenerative diseases (Manservigi et al., 2010; Poliani et al., 2001; Wolfe et al., 2004a).

An adenovirus mutant has recently been approved in China for use in patients with head and neck squamous cell carcinoma (Crompton and Kirn, 2007). In the United States, nearly 20 ongoing or completed phase I and II clinical trials of oncolytic virotherapy using derivatives of at least seven virus types including HSV, vaccinia, Seneca Valley virus, Coxsackie virus, reovirus, measles virus, and NDV have been documented (clinicaltrials.gov, March 7, 2010; Hammill et al., 2010).

NDV is one such virus with an inherent oncolytic property. In humans, it is reported to have oncolytic and immunostimulatory effects (Dembinski et al., 2010). It specifically replicates in tumor cells while sparing normal cells and causes oncolysis (Ravindra et al., 2009). The oncolytic efficacy of NDV is mediated through its ability to selectively lyse tumor cells by apoptosis (Elankumaran et al, 2006) and the apoptotic ability is mediated through its HN protein (Ravindra et al., 2008). Tumor response and prolongation of survival have been demonstrated in several models after single or multiple injections, with complete tumor regression in some cases by using HSV-1 (Harland et al., 2002; Randazzo et al., 1995). A variety of RNA viruses have been studied as possible cancer therapies including reovirus. Reovirus is intrinsically oncolytic without any genetic exploitation. The inherent oncolytic properties of this virus are consequent from the fact that it specifically targets cells with an activated Ras pathway which is found in many cancer cells (Kapadia and Coffey, 2010).

Despite the advantages of replicating OV_s, their use in virotherapy is debatable in cancer patients with respect to their possible disease inducing capabilities. Hence, the consensus is to use non-human viruses and non-replicating OV_s that can be relatively safer to use in immunocompetent and immunocompromised patients. Different types of defective recombinant vectors have been developed in the past years and a few factors have to be taken into consideration for their design. The following criteria have been followed in order to create an efficient replication defective OV: By eliminating the lytic viral gene expression and also the innate immune responses that are toxic to the host; by engineering the promoter systems so as to accomplish most suitable, long-term transgene expression; by synchronized expression of various genes (Burton et al., 2002; Manservigi et al., 2010). Non-replicating OV_s can also be engineered in the laboratory as gene delivery vehicles that have anticancer activity (Aghi and Chiocca, 2006). Amongst the OV_s used for virotherapy; paramyxoviruses are capable of forming non-replicating OV_s more efficiently and have been used in several clinical trials (Hammill et al., 2010).

2.5 Virus Like Particles

Virus-like particles (VLP_s) resemble viruses, but are non-infectious because they do not contain any viral genetic material. The expression of viral structural proteins, such as envelope and capsid proteins can result in the self-assembly of VLP_s which mimic the conformation of a native virus. VLP_s are incapable of multiple rounds of infection, yet they retain the superb antigenicity of virus particles. VLP_s are commonly used in studies to identify protein components required for viral assembly and also as a useful tool for the development of vaccines. VLP vaccines can be produced relatively rapidly; within weeks as compared to months for egg-produced vaccines, which make them particularly useful when new pandemic strains emerge as in influenza viruses. VLP_s contain viral surface proteins, which offer conformational viral epitopes that can elicit strong T and B cell immune responses (Akahata et al., 2010).

Most paramyxoviruses are able to self-assemble as VLPs. The requirements for budding and release of non-infectious, non-replicating VLPs for many paramyxoviruses have been documented (Alexander, 2000; McGinnes et al., 2010; Pantua et al., 2006). The M protein of most paramyxoviruses is sufficient for the release of VLPs from transfected cells even in the absence of other proteins. Membrane deformation and vesicle budding have been reconstituted *in vitro* using purified M protein and unilamellar vesicles in NDV (Shnyrova et al., 2007), representing that all of the actions required for inducing curvature and fission of a membrane are enclosed within M protein. It has been shown in NDV VLP studies, that only M protein was sufficient for NDV like particle release with an efficiency that was similar to that observed when all four structural proteins were expressed, implying that no other protein is required for efficient release of VLPs (Pantua et al., 2006).

The critical role of M protein in paramyxovirus assembly has been obtained through study of viruses with mutations in M protein (Harrison et al., 2010). Early studies were dependant on Sendai virus temperature-sensitive mutants in which M protein was unsuccessful in accumulating to threshold levels at non-permissive temperatures. This failed to produce virus particles (Kondo et al., 1993; Yoshida et al., 1979). Studies have demonstrated compelling efficient paramyxovirus particle formation only in the presence of a threshold level of functioning M protein (Cathomen et al., 1998b; Harrison et al., 2010; Inoue et al., 2003). VLP production from transfected cells with M protein becomes more efficient when the M proteins are co-expressed with other viral components, such as glycoproteins, nucleocapsid proteins, and C proteins (Harrison et al., 2010). Some paramyxovirus M proteins lack the capacity for directing efficient VLP production when expressed alone in cells. Hence, the requirements for efficient VLP production differ among paramyxoviruses. However, for paramyxovirus VLP production, M protein appears to be the major requirement whether expressed alone or in combination with other glycoproteins (Li et al., 2009; Schmitt et al., 2002; Teng and Collins, 1998).

VSV budding depends on a functional cellular VPS4 protein whereas influenza virus VLP formation occurs independent of a functional VPS4 protein (Bruce et al., 2009). M protein appears to be the major requirement for most paramyxovirus VLP production. VLPs failed to form in the absence of M protein (Li et al., 2009; Schmitt et al., 2002; Teng and Collins, 1998) whereas for hemagglutinin (HA) glycoprotein is the major requirement for influenza A VLP budding (Chen et al., 2007). Hepatitis E Virus like particles are not yet attainable by optimal cell culture system, but there has been a good progress with the *in vitro* expression of HeV-like particles that required ORF3 domain for efficient budding of HeV particles (Bihl et al., 2010; Bihl and Negro, 2010; Chandra et al., 2010). For Ebola virus (Jasenosky and Kawaoka, 2004; Jasenosky et al., 2001), VSV (Jayakar et al., 2004), MeV, hPIV-1, NiV, Sendai virus (Sugahara et al., 2004; Takimoto et al., 2001) and NDV (Pantua et al., 2006), M protein is sufficient for VLP production. In PIV5, M protein expressed alone does not result in VLP production (Coronel et al., 1999); F and HN proteins are necessary but redundant for VLP production whereas in MuV (Li et al., 2009), M protein expressed alone does not result in efficient VLP formation but along with F it is enhanced (Schmitt et al., 2002; Subhashri and Shaila, 2007).

2.5.1 Virus-based nanoparticles for cancer therapy

With the beginning of nanotechnology, nanoparticles have emerged as effective agents to carry drug payloads to specific sites, permitting localized activity as well as payload protection (Farokhzad and Langer, 2009; Narayanan et al., 2010). The term “nanoparticles” was coined due to particle sizes being extremely small and covering a range between 100 and 2500 nanometers in diameter. They have been extensively used for antigen delivery and efficient activation of cells in the immune system owing to their ease of manufacture, to administer, ability to be well tolerated, and dispense with the need for exogenous adjuvants. Nanoparticles are immunogenic and when injected into animals, they induce the production of antiviral antibodies that can block infection.

Nanoparticles have also gained considerable recognition as new-generation vaccines that are safe and effective.

Targeting of tumors by conjugating nanoparticles with targeting ligands against tumor-cell-specific receptors for receptor mediated endocytosis, is possible (Narayanan et al., 2010). Ideally, specific delivery of drug-loaded nanocarriers to the area of interest would grant the maximum therapeutic efficacy (Torchilin, 2007). There have been several attempts in using folic acid (FA) and other cell-type specific ligands for exploring the advantages of targeted delivery of chemotherapeutic phytochemicals on multiple cancer cells without any known lethal effects on normal cells. The FA owing to its smaller size, lack of immunogenicity, high stability, ready availability and low cost has proven itself as a proficient targeting moiety. Importantly, folate receptor (FR) is highly expressed in several types of solid tumors such as ovarian, uterine, lung, breast, and head and neck cancers (Narayanan et al., 2010; Wang et al., 2009). In contrast, normal tissues lack FR expression, making folate an exceptional tumor-targeting moiety. It has been reported that FA is taken up by FRs which are glycosyl–phosphatidylinositol linked membrane proteins, by a theorized process known as “potocytosis” (Farokhzad and Langer, 2009).

The exogenous administration of viral-derived proteins are used to stimulate tumor-selective cell death on virus based therapies as demonstrated for the adenovirus-derived E4orf4, the parvovirus H1 protein NS1 and chicken anemia virus-derived apoptin (Guelen et al., 2004; Maddika et al., 2005; Maddika et al., 2006; Rohn and Noteborn, 2004). Chicken anemia virus-derived apoptin induces apoptosis in a wide variety of human cancer cell lines via classical apoptotic pathways (Backendorf et al., 2008; Maddika et al., 2005; Maddika et al., 2006; Rohn and Noteborn, 2004).

A few prophylactic VLP-based vaccines like Glaxo SmithKline's *Engerix* (hepatitis B virus) and *Cervarix* (human papillomavirus), and Merck and Co., Inc.'s *Recombivax HB* (hepatitis B virus) and *Gardasil* (human papillomavirus) are at present commercialized globally (Roldao et al., 2010). Other VLP-based vaccine candidates such as influenza virus, parvovirus, Norwalk and various chimeric VLPs are currently in clinical trials or undergoing preclinical evaluation. Many other VLPs are still controlled to small-scale fundamental research, despite their accomplishment in several preclinical tests (Barra et al., 2010; Roldao et al., 2010).

VLPs can be targeted by employing strategies that can cause particle activation by cancer-specific proteases; targeting by entry through cancer-specific cell-surface molecules and by exploiting cancer-specific molecular defects as described for OV_s (Cattaneo et al., 2008). Viral particles can be modified by targeting cancer cells expressing proteases like matrix metalloproteases (MMP); by conjugating to cell-targeting ligands by antibody–virus interactions, or by molecular bridges like biotin–avidin and chemically by polyethylene glycol (PEG) (Cattaneo et al., 2008). Cancer cells expressing folate receptors can also be targeted by VLPs conjugated with FA. VLPs are capable of incorporating therapeutic payloads effectively and hence remodeling of VLPs using critical proteins and by combination with other proteins, drugs, apoptotic genes, etc., makes them more popular to use in vaccine therapy and cancer therapy. Many OV_s can efficiently form VLPs when cultivated experimentally in vitro, therefore, combining the oncolytic properties of OV_s with their ability to incorporate various therapeutic payloads in VLPs makes their use a success in most cases. Also, genetic manipulation of most OV_s has restrictions for the size of the therapeutic cargo that they can hold.

NDV VLPs have the ability to fuse and modulate their surface with the same effect in host cells as the authentic virus particles (Singh et al., 2010). The cytoplasmic tail (CT) and transmembrane (TM) domain of NDV HN and F proteins are easily modifiable and they have the

ability to incorporate foreign genes or proteins in the CT and TM (McGinnes et al., 2010) and there has been evidence that the CT and TM of HN and F interact with M protein of NDV (McGinnes et al., 2010; Murawski et al., 2010). This ability of NDV envelope glycoproteins CT and TM affords a great advantage to incorporate foreign antigens such as therapeutic transgenes (pro-apoptotic genes, cytokines); tumor antigens etc., besides the capability to surface decorate for ligand-directed targeting. Besides, the inherent ability of the NDV HN protein to induce apoptosis independent of virus replication (Ravindra et al., 2008) offers a novel approach to deliver HN through the VLP platform dispensing with the need for virus replication in the host cell and associated toxicity issues in immunocompromised hosts. Further, the cell specific delivery of NDV glycoproteins in cancer cells would usurp the host immune system to redirect the antiviral immunity toward VLP-infected tumor cells. These novel NDV-based non-replicating platforms could serve both as non-replicating OVs and as immunotherapeutic cancer vaccines.

In general, OVs hold great potential as strong, self-amplifying cancer therapeutics (Cattaneo et al., 2008). Virotherapy is a very promising and attractive strategy because there is no cross-resistance with chemotherapy and radiation therapies and with the advent of VLP based platforms for cancer vaccines and virotherapy, it assures the use in immunocompromised patients. The ease with which the VLPs can be manipulated has drawn a number of groups to exploit their capabilities in the world of virotherapy for cancer research (Cattaneo, 2010; Kelly and Russell, 2007).

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2.7 Figure

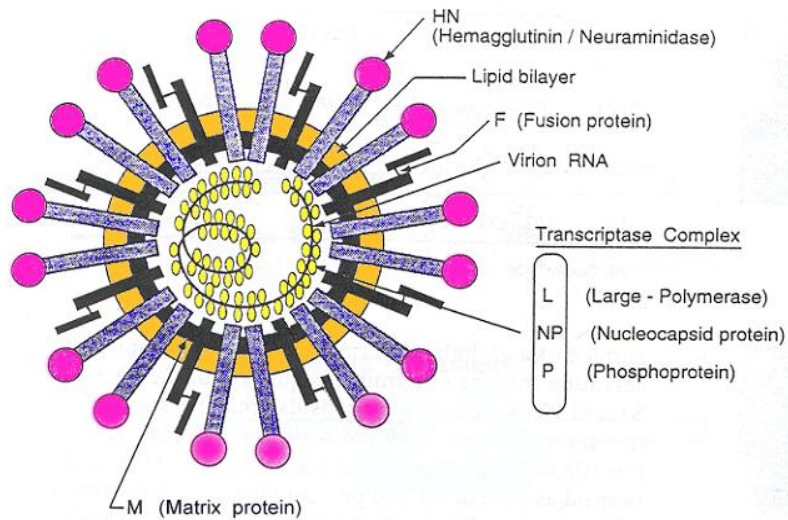


Figure 2.7. Diagrammatic representation of Newcastle disease virus: Fusion protein (F) and attachment (HN) proteins appear as spikes on the virion surface. Matrix protein (M) inside the envelope stabilizes virus structure. The ribonucleocapsid core is composed of the genomic RNA, nucleocapsid protein (NP), phosphoprotein (P) and Large polymerase protein (L) forming the transcriptase complex (Source: Dr. Elankumaran Subbiah).

Chapter 3: Virus-based Nanoparticles for Tumor Selective Targeting and Oncolysis

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3.1 Abstract

Many oncolytic virotherapies have shown great advantages for rapid, rational design through recombinant DNA technology to facilitate the targeting of a broad spectrum of malignancies. Ligand-directed, receptor-mediated targeting is a common platform for targeting oncolytic viruses (OV) to specific cell types. Our approach is to develop folate-receptor targeted Newcastle disease virus (NDV) based nanoparticles (VBNP) for oncolytic virotherapy. NDV, an avian paramyxovirus, is naturally tumor-selective and inherently oncolytic. We have devised a technique for obtaining VBNPs by co-expressing matrix (M), hemagglutinin (HN), and fusion (F) proteins of the virus in avian/ mammalian cells. VBNPs are known to be non-infectious, lacking in viral genome, non-replicating and kill a variety of tumor cells by apoptosis. We found that the budding characteristics and morphology of VBNPs were similar to authentic NDV virions. The VBNPs ranged in size from 50nm to 300nm. We also engineered VBNPs to express the apoptin (VP3) gene of chicken anemia virus, which confirmed to be cytotoxic to tumor cells. VBNPs were engineered to specifically target folate-receptor bearing tumor cells by surface conjugation to folate. Analysis of cytokine profiles induced by VBNP and VBNP-VP3 in tumor cells showed

an induction of proinflammatory and chemotactic cytokines. Regression studies of xenotransplanted human HT1080 fibrosarcoma and U87MG malignant glioma in nude mice using multiple doses of VBNP, VBNP-VP3 and VBNP-VP3-Fo on days 0, 6, 18, 28 after tumor induction showed a propensity for limiting tumor growth during the time of treatment in HT1080 injected tumors. The VBNP treated animals also had a slight survival advantage over the control group in HT1080 injected tumors although no statistical significance was seen. Our results suggest that VBNPs could be used to deliver small molecules, anti-tumor/reporter genes and also aid in generating tumor specific immunity by rational design.

3.2 Introduction

Oncolytic virotherapy for cancer treatment has gained increasing recognition by the use of oncolytic viruses that can be engineered to selectively replicate within cancer cells and thus kill tumors. The cells that were transformed with oncogenes or which were in active cell-cycling state allowed replication of some viruses (Farassati et al., 2001; Imperiale et al., 1984). These viruses are tumor-specific, self-replicating, lysis-inducing cancer killers that give hope and in the future it may be possible to completely eliminate certain types of tumors at local, regional, and distant sites using a single treatment (Davis and Fang, 2005).

The efficacy of OVs may be limited by their immunogenicity. Although immunosuppression can improve viral oncolysis (Bridle et al., 2009), the host's immune system responds by efficiently neutralizing and inhibiting viral vectors from having any prolonged persistence or widespread distribution. OVs for replication must produce viral proteins intracellularly, which are processed and presented on major histocompatibility type I (MHC-I) proteins as viral antigens. These complexes naturally attract cytotoxic T lymphocytes (CTL), which efficiently clears intracellular pathogens, especially viruses (Neff-LaFord et al., 2003) by randomly destroying any cells exhibiting these antigens, thus eliminating cell populations that harbor therapeutic OVs. Viral infection interferes with the immune tolerance some tumors induce (Fuchs and Matzinger, 1996; Ochsenbein et al., 1999). As a result, attracted CTLs specificity against the tumor antigen will support proliferation and attack cells that present the tumor antigen. Theoretically, this same outcome could be reconciled with non-replicating, transgene-expressing vectors (Asemissen et al., 2005; Stevenson et al., 2004; Wodarz, 2001).

Like with any clinical treatment, questions have and will persist in oncolytic virotherapy (OVT). Nevertheless, some efforts have been made to prevent or thwart the effects of viral

infection and improve the efficacy, safety and applicability of OVT. Immunosuppressed or immunodeficient patients are highly susceptible to uncontrollable viral infections. Even when restricted by several mechanisms, no gene therapy, especially replicating viruses, can be completely guaranteed to adhere to its restrictions. Hence, in order to counteract this problem in virotherapy, the use of non-replicating OVAs has gained considerable popularity. Owing to their relatively safer use in immunocompromised patients and ease of manipulation to carry therapeutic payloads on their surface, non-replicating OVAs can be used in conjunction with radiation and chemotherapy, reducing their doses considerably (Davis and Fang, 2005).

Virus-like particles (VLP), which are non-infectious and non-replicating, are progressively getting accepted as safe, effective vaccines (Jennings and Bachmann, 2008). VLPs are virus-sized particles composed of recurring structures on their surfaces and in their cores, which impersonate those of infectious viruses that accounts for the very effective immunogenicity of viruses (Jennings and Bachmann, 2008; Rice et al., 2004; Stevenson et al., 2004). VLPs are formed by the assembly of the structural proteins and at times lipids without the integration of the viral genome. Thus, VLPs are inept at several rounds of infection, but preserve the superb antigenicity of virus particles (Wodarz, 2001). VLPs permit the insertion or fusion of foreign antigenic sequences, resulting in chimeric particles that can deliver foreign antigens on their surface including non-protein antigens via chemical conjugation (Buonaguro et al., 2010). This could be a safety net, to provide control over the use and effect of VLPs in cancer patients.

Newcastle disease virus (NDV), an avian paramyxovirus, is a single-stranded, negative sense RNA virus. NDV is naturally tumor-selective and inherently oncolytic and has shown promise in several preclinical models and in human clinical trials (Lech and Russell, 2010; Wu et al., 2010b). Avian cells that express viral NP, M, HN and F proteins are capable of releasing

VLPs which have similar protein ratios to infectious virus with homogeneous densities which differ only slightly from the authentic virus (Pantua et al., 2006).

The folate receptor (FR) is a glycosylphosphatidylinositol (GPI)-anchored, high-affinity, membrane binding protein, which is over-expressed in a wide variety of human tumors (Ross et al., 1994). In the interim, FR distribution in normal tissue is highly restricted, making it a useful marker for targeted drug delivery to tumors. Folic acid (FA), a high-affinity ligand for the FR, retains its receptor binding property when covalently derivatized via its gamma-carboxyl terminal (Cheng et al., 2009). Conjugation of targeting ligands onto nanocarriers enables selective delivery of OV's to improve therapeutic responses both *in vitro* and *in vivo* (Yu et al., 2010). Folate conjugated poly-L-lysine coupled with replication-defective adenovirus has been shown to mediate receptor-specific transfection *in vitro* in tumor cells (Gottschalk et al., 1994).

We hypothesized that non-replicating, non-infectious virus-based nanoparticles (VBNPs) that are selectively targeted and armed could be engineered using NDV. To obtain proof-of-concept for our hypothesis, we generated VBNPs using NDV structural proteins and evaluated their oncolytic efficacy *in vitro* and *in vivo*. The VBNPs were further engineered to express the apoptin (VP3) gene of chicken anemia virus and subsets of them were targeted to folate-receptor bearing tumor cells by surface conjugation to folate. Targeted and non-targeted (Fo+, Fo-), armed or unarmed (VP3+, VP3-) VBNPs were found to be cytotoxic to tumor cells by apoptosis. Folate-targeted VBNPs were capable of infecting only folate-receptor (FR+) bearing cells. The VBNPs were able to elicit pro-inflammatory cytokine and chemotactic chemokine response in tumor cells. Although the VBNPs failed to completely regress xenotransplanted human fibrosarcoma and malignant glioma in Balb/c nude mice, they were able to initiate tumor cell death and marginally prolong survival in treated animals, suggesting the need for dose optimization. Our results favor the development of NDV VBNP platform for oncolysis.

3.3 Materials and Methods

3.3.1 Cells: DF1 chicken embryo fibroblast cells, HT1080 fibrosarcoma cells and 293T human epithelial kidney cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin-streptomycin (Gibco Inc.) and 10% fetal calf serum (FCS) (Thermo Scientific). U87MG, human glioma and A549, human lung carcinoma cell lines required MEM (Gibco Inc.) and RPMI 1640 medium (Gibco Inc.) respectively, with 10% FCS and penicillin-streptomycin (Gibco Inc.). SVHUC, SV40 Immortalized Human Uroepithelial Cells were grown and maintained in DMEM/F12 (Gibco Inc.) + 10% FCS and penicillin-streptomycin. Cells were incubated at 37°C in a humidified incubator with 5% CO₂. For infectivity assays, a maintenance medium containing the specific medium for the cell lines with 2% FCS and antibiotics was used.

3.3.2 Plasmids: NDV cDNA sequences encoding M, HN, and uncleaved F proteins were subcloned into the expression vector pCAGGS (Pantua et al., 2006) to generate pCAGGS-M, pCAGGS-HN, and pCAGGS-F, respectively.

3.3.3 Generation of VBNPs: Transfection of sub-confluent DF-1 cells was accomplished using Lipofectamine (Invitrogen Inc.) as recommended by the manufacturer. The following amounts of plasmid DNA were used per 150-mm dish: 10 µg pCAGGS-M, 10 µg pCAGGS-F, and 10 µg pCAGGS- HN. For each transfection, a mixture of each of the three plasmids above with 2 mL OptiMEM medium (Invitrogen Inc.) and 10 µl of Plus reagent (Invitrogen Inc.) in tube A and 2 mL OptiMEM medium with 20 µl Lipofectamine 2000 in Tube B were incubated at room temperature for 20 min. Tube A and B were mixed and further incubated for 20 min at room temperature. Then, 4 mL of this mixture was added to each 150-mm dish after washing it 3

times with phosphate buffered saline (PBS, pH 7.4) and further incubated at 37°C, 5% CO₂ for 4-6 h. After incubation, 16 mL of DMEM with 2% FCS and antibiotics was added to each dish. At 72 h post-transfection, cells were harvested with a cell scraper and clarified at 5000g for 30min. The supernatant was collected and subjected to ultracentrifugation.

3.3.4 Generation of VBNP with Apoptin: To generate VBNP with chicken anemia virus apoptin (VP3) gene, apoptin was fused to NDV-F gene transmembrane (TM) and cytoplasmic domains (CT) and cloned into pCAGGS expression vector (Fig. 1). The primers were designed for a 2-step PCR reaction and the resulting clone (VP3) was inserted into pCAGGS using NheI and ECoRI restriction sites. The primers used were: 5'- **GCTAGCTCACATTTTGTAGTGGC**-3' (forward) and 5'- **GAATTCGCCACCATGAACGCTCTCCAAGAAGATACTC**-3' (reverse) (letters in boldface represent NheI and ECoRI restriction enzyme sites, respectively). The VBNP-VP3 was obtained by transfecting the pCAGGS-VP3 and pCAGGS-F, pCAGGS-HN and pCAGGS-M plasmids as above. The VBNP-VP3 containing supernatants were clarified and subjected to purification by ultracentrifugation.

3.3.5 VBNP purification: The clarified supernatants were pelleted by centrifugation in a SW28 rotor in a Beckman ultracentrifuge (Beckman Inc., USA) at 25,000 rpm for 2 h at 4°C. The pellet was re-suspended in TNE buffer (25 mM Tris- HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) (Pantua et al., 2006) and overlaid on top of a step gradient consisting of 5 mL 20% and 3 mL 55% sucrose solutions (g/mL) in Ca/Mg free PBS and centrifuged using a SW41 rotor at 22,000 rpm for 2 h at 4°C. The band at the interface (containing concentrated particles) was collected and was diluted in TNE buffer and centrifuged in a SW28 rotor at 25,000 rpm for 2 h at 4°C for

concentrating the VBNPs. The pellet was then re-suspended in 500 µl of TNE buffer and stored at 4°C until use. The purified VBNPs were further filtered through Amicon Ultra-15 centrifugal filter (Millipore Inc.) at 5000 xg for 15 minutes. The retentate was collected containing the concentrated and purified VBNPs and was later dialyzed overnight with constant stirring using a Slide-A-Lyzer Dialysis Cassette (Pierce, USA) in PBS, pH 7.4.

3.3.6 Folate conjugation of VBNPs: The VBNPs were conjugated to N-hydroxysuccinimide ester of folate (*NHS-folate*) and NHS-fluorescein (Thermo Scientific, USA) (Molar ratio 1:1) and then dialyzed using a Slide-A-Lyzer Dialysis Cassette (Pierce, USA) in PBS, pH 7.4 overnight. For *in vivo* experiments, the VBNPs were conjugated only to folate using NHS-folate. The protein concentration of folate and folate/fluorescein conjugated VBNPs was measured by Micro BCA protein assay kit (Thermo Scientific, USA).

3.3. 7 Cell entry and subcellular localization of VBNPs: To determine the cell entry of VBNPs, DF-1 cells were grown in chamber slides (Lab-Tek) and layered with 100 µl (0.25 ng/mL) of VBNPs. After 72 h at 37°C, the nuclei were stained with 5 µg/mL of Syto-60 (Invitrogen Inc.) for 10 min at 37°C. Cells were then washed two times with ice-cold immunofluorescence (IF) buffer (PBS containing 1% bovine serum albumin, 0.02 % sodium azide, and 5 mM CaCl₂), fixed with 2% paraformaldehyde, blocked with IF buffer for 2 h, and incubated for 1 h at 4°C in IF buffer containing polyclonal chicken antiserum against NDV (SPAFAS, Inc, USA) at 1:500 dilution. Cells were then washed two times with ice-cold IF buffer, followed by incubation for 1 h at 4°C in IF buffer containing fluorescein conjugated goat anti-chicken secondary antibodies (KPL). The cells were then washed two times with ice-cold IF

buffer and stained with Syto-60 (Molecular Probes, Invitrogen inc.) as a nuclear stain for 5 min. The cells were washed and mounted with 1:1 glycerol: methanol for immunofluorescence microscopy. Fluorescence images were acquired using a Nikon eclipse TE 2000-E confocal microscope (Nikon, Inc., USA).

For demonstrating FR-mediated uptake of VBNPs, chamber slides (Lab-Tek) were plated with HT1080 and 293T cells and layered with 100 μ l (0.25 ng/mL) of FA and fluorescein conjugated VBNP. After 2 h incubation at 37°C, 5% CO₂, the cells were washed once with PBS, pH 7.4 and fixed using 1:1 methanol-acetone for 45 min. The cells were washed again with PBS, pH 7.4 three times before staining the nucleus with DAPI (4', 6-diamidino-2-phenylindole) (Molecular Probes, Invitrogen Inc.). The slides were mounted on 1:1 glycerol-PBS and visualized under a Zeiss LSM 490 confocal microscope.

3.3.8 Transmission Electron Microscopy: 293T cells were plated in 6 well plates and transfected using 1 μ g of each pCAGGS-M, -F, -HN plasmids. The transfected cells were harvested and centrifuged at 3000 rpm in a microcentrifuge (ThermoScientific Inc.) for 5 min to form a loose pellet. Pellet was fixed overnight in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). The pellet was washed using cacodylate buffer, and incubated in 2% OsO₄ for 2 h. After washing with water, the sample was stained overnight in 0.5% uranyl acetate, washed again and dehydrated through a graded acetone (50%, 70%, 90% and 100% acetone, 5 min each) series. The sample was embedded in formvar resin and ultrathin sections (80 nm) were stained with uranyl acetate and lead citrate. The sections were overlaid on 200 mesh carbonated nickel grids (Electron Microscopy Sciences, PA). The morphologic characteristics and budding of VBNPs were examined using a Philips 420 transmission electron microscope.

3.3.9 Immunogold Transmission Electron Microscopy: Purified VBNPs (10 µl) was adsorbed on to carbon coated 200 mesh gold grids (Electron Microscopy Sciences, PA) and incubated for 1 h at 4°C in a humidified chamber to enrich the VBNPs on the grid. The grids were blocked with PBS, pH 7.4 containing 1% BSA. Adsorbed VBNPs were then stained with polyclonal anti-NDV chicken serum (SPAFAS, Inc.) at a dilution of 1:10. The bound antibody was probed with 12 nm colloidal gold with donkey anti-chicken IgY antibody (Jackson Immunoresearch), followed by negative staining with 2% phosphotungstic acid (PTA), pH 6.6. The stained grids were examined under a Philips TEM 400 transmission electron microscope at 55,000 x magnification.

To visualize the structure of VBNPs, purified VBNPs were adsorbed onto carbon coated 200 mesh gold grids (Electron Microscopy Sciences, PA) and were subjected to negative staining with 2% PTA as above. The stained grids were examined for VBNPs under a Philips TEM 400 transmission electron microscope at 55,000 x magnification.

3.3.10 Immunoblotting: The sucrose purified VBNPs were subjected to immunoblotting using standard procedures. Proteins were separated in a 4-20% sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P (Millipore) membrane. The membrane was blocked overnight in 5% skimmed milk and 0.05% Tween 20 solution at 4°C. The membrane was washed in PBS, pH 7.4 with 0.05% Tween 20 (PBST) and incubated at 4°C with 1:500 dilution polyclonal anti-NDV chicken serum (SPAFAS, Inc) for 1 h. The membrane was washed 3 times on a shaker plate with PBST for 10 min each before labeling

the membrane with 1:5000 dilution of horseradish peroxidase labeled anti-chicken antibody (KPL) for 1 h. The membrane was washed 3 times with PBST for 30min. Then, 2mL of chemiluminescence TMB substrate (KPL) was added and allowed to react with the membrane. The enzyme-substrate reaction was stopped with water before visualizing the protein bands under ChemDoc XRS (Thermo Scientific Inc.).

3.3.11 Coomassie blue staining for VBNP composition: The proteins from sucrose purified VBNPs were separated in a 4-20% sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and later the gel was fixed in 10 volumes of 1X fixer for 30 min at room temperature. Fixer (1X) was prepared by mixing 40% methanol and 10% acetic acid. After discarding the fixer, gel was stained in 10 volumes of Coomassie Blue Stain for 1 h at room temperature or when the bands appear distinct. Destaining of the gel was done by using 10 volumes of 1X destain containing 50% methanol in water with 10% acetic acid for 30 minutes at room temperature. Gel was soaked in distilled water for 15 min before visualizing the gel under Chemdoc XRS (ThermoScientific).

3.3.12 Cytometric bead array (CBA): Human tumor cells such as HT1080 fibrosarcoma and U87MG malignant glioma cells were grown in 6 well plates (Corning Inc.) and infected with 100 μ l (0.25 ng/mL) of VBNP. Cell supernatant was collected at 72 h post infection and clarified by centrifugation. Using the human CBA flexi kit (BD Biosciences Inc.), the cells were then processed and analysed for different cytokines and chemokines like IFN α , IL-6, IL-8, MIP-1 α , MCP-1 and RANTES by flow cytometry following manufacturer's guidelines.

3.3.13 WST-1 assay: It is a colorimetric assay for analyzing cell viability and is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases. WST-1 assay was performed essentially as described (Elankumaran et al., 2006). In each experiment, the tumor cell line was seeded into 96-well plates at 1×10^4 cells/well in DMEM containing 10% FCS and antibiotics (Invitrogen, USA). Following overnight incubation (37°C and 5% CO₂), the medium was removed by aspiration, and washed with PBS, pH 7.4 once before adding 50 µl of 0.25 ng/mL and 0.025 ng/mL of VBNP or VBNP-VP3 to the wells. After 2 h incubation, 100 µl of medium was added and further incubated for 72 h. The cell viability was measured by adding 10 µl of WST-1 reagent (Roche, USA) in each well. An expansion of the number of viable cells increases the overall activity of mitochondrial dehydrogenases. The formazan dye produced by metabolically active cells was quantified 2 h after the addition of WST-1 reagent by measurement in a multiwell spectrophotometer at 450 nm (Tecan Sapphire 2) and Magellan 6 software. Background absorbance was subtracted using the control medium and WST-1 reagent. The control cells were normalized to 100% cell viability.

3.3.14 Flow cytometry: Tumor cells were grown *in vitro* in tissue culture treated 6 well plates (Corning Inc.) and infected with 100 µl (0.25 ng/mL) of VBNP or VBNP-VP3. The infected cells were incubated for 48 h and were trypsinized, centrifuged and resuspended in 500µl 1X binding buffer. From each cell suspension, 2×10^5 cells were centrifuged and resuspended in 500 µl 1X FACS binding buffer and then stained with 5 µl Annexin V conjugated to FITC or Pacific Blue (BD Biosciences), respectively and 1 µl Propidium Iodide (PI, 1mg/mL) to each well (BD Biosciences) and incubated for 5 min in dark. The percentage of cells undergoing apoptosis was

determined by flow cytometry (FACS Calibur System, BD Biosciences, Franklin Lakes, NJ). The annexin-V-FITC positive and PI-negative cells were enumerated as early apoptotic cells.

3.3.15 Apoptotic DNA laddering: DF-1 cells (2×10^6 cells) were infected with 100 μ l (0.25 ng/mL) of VBNP and trypsinized using 0.025% Trypsin EDTA (Gibco Inc.) and washed with PBS, pH 7.4. Intranucleosomal DNA fragmentation in VBNP infected cells was detected by using an apoptotic DNA laddering kit (Roche), following the manufacturer's instructions.

3.3.16 *In vivo* tumor models: All procedures involving animals were in accordance with NIH protocols and were executed according to the guidelines of the Institutional Animal Care and Use committee (IACUC) of the Virginia Polytechnic Institute and State University. Six-week-old Balb/c nude mice (Harlan) were housed in groups of 3 in micro-isolator cages under pathogen-free conditions at the Integrated Life Sciences Building (ILSB) vivarium. HT1080 human fibrosarcoma (5×10^6 cells/ 100 μ l) and U87MG human malignant glioma cells (5×10^6 cells/ 100 μ l) were implanted subcutaneously into the flank of mice. Tumors reaching more than 5 mm in diameter were intratumorally inoculated with 50 μ l (6 μ g/100 μ l, 0.6 μ g/100 μ l concentrations) of VBNP, VBNP-VP3 or VBNP-VP3-Fo on day 0, followed by 100 μ l of each on days 6, 18, and 28. These two concentrations of VBNPs used are referred to as VBNP, VBNP 1/10, VBNP-VP3, VBNP-VP3 1/10, VBNP-VP3-Fo, VBNP-VP3-Fo 1/10 hereafter in the text. Tumor volumes were measured in two planes and body weight was monitored every 2 days. The tumor volume was calculated using the formula: Tumor volume= $(L \times B^2)/2$, where "L" is the length of the longest diameter in cm, "B" is the length of the shortest diameter in cm. Animals were euthanized

when tumor volume reached >20 % body weight or at defined experimental time point (Day 60) following the IACUC protocol.

3.3.17 Histopathology: For histological analyses, tumor tissue and spleen were collected from each mouse at necropsy and fixed in 10% formalin and processed in paraffin, sectioned, and stained with hematoxylin and eosin (H/E) at the Histopathology unit, Virginia-Maryland Regional College of Veterinary Medicine. The tumor sections were examined and scored based on the degree of necrosis by a board-certified veterinary pathologist (T. LeRoth) blinded to the experimental groups.

3.3.18 Statistical analysis: The tumor volume was analyzed by one-way analysis of variance (ANOVA), Student's t- test and Tukey's test in JMP 8.0 software. The tumor volume of each mouse in a group was calculated on treatment days until Day 42 post treatment. Significance of tumor volumes between the treatment groups was analyzed by Dunnett's test. A *P* value of ≤ 0.05 was considered to be significant. Survival curves were plotted by Kaplan-Meier method and differences between groups were investigated using the log-rank test in JMP 8.0.

3.4 Results

3.4.1 Morphology, budding and release of VBNP: The cells expressing M, F and HN proteins of NDV were able to produce VBNPs with morphological characteristics reminiscent of authentic NDV virions. DF-1 cells were transfected with M, F and HN plasmids and were observed every 24 h. Multinucleated cells in the form of syncytia were evident in DF-1 cells 48 h post-transfection (Fig. 2). Similarly, VBNP-VP3 plasmid was transfected into DF-1 cells with M, F, HN and VP3 plasmids and fusogenicity was observed 48 h post transfection (Fig. 3). The co-expression of M, F and HN proteins resulted in the formation of VBNPs reminiscent of the infectious virus but lacking the nuclear core material. The VBNPs displayed generally spherical morphologies and densely stained cores. The spike projections on the surface of VBNPs were no different in appearance to those on authentic NDV virions.

To study the assembly and budding of VBNPs, 293T cells were transfected with M, F and HN plasmids. From TEM images, it was confirmed that the VBNPs assembled in the cytoplasm of the cell and budded out from the cell membrane. Pleomorphic VBNPs were also observed after budding and release from the cell membrane, having a distinct viral envelope and had dense material packed in the core (Fig. 4A to C). It appears that the VBNPs packed the cellular material during assembly and budding from the cell wall. Recently, it has been shown influenza A virus VLPs incorporated several cellular proteins. The resemblance between VLP proteome and virus cellular proteins suggested that the budding behaviors and constituents of mammalian VLPs are very similar to those of authentic viruses (Wu et al., 2010a) (Fig. 5A to C).

The presence of NDV F and HN glycoproteins on the surface spikes of these VBNPs were confirmed by using immunogold labeled with polyclonal anti-NDV chicken antibody and counterstained with 12 nm gold spheres coupled to secondary antibodies (Fig. 6).

To determine if the purified VBNPs were composed of HN, M and F proteins, the purified VBNPs were subjected to SDS-PAGE and confirmed by immunoblot which detected the HN at ~75K Da., M at ~37K Da., and F at ~50K Da (Fig. 7A to B). To determine whether the VBNPs packed the cellular material during assembly to form a particle, purified VBNPs and VBNP-VP3 were subjected to SDS-PAGE followed by Commassie Blue staining of the gel. It was found that several non-viral and possibly cellular proteins were packed into the VBNPs (Fig. 7C), before budding out from the cell. Immunoblot of the corresponding gel confirmed the presence of NDV glycoproteins and M protein in the VBNPs (Fig. 7A to B).

3.4.2 Folate conjugated VBNP uptake in Folate receptor (FR) positive cell line: To determine whether folate conjugated VBNPs are taken up only in cells that expressed FR on their surface, HT1080, fibrosarcoma cells and 293T, human epithelial kidney cells were used to represent the FR negative (FR-) and FR positive (FR+) cell lines, respectively. Confocal microscopic analysis confirmed the uptake of folate conjugated VBNPs in FR+ cell line (293T) and not in FR- cell line (HT1080), showing selective uptake and targeting of FR (Fig. 8A to B).

3.4.3 Cytoplasmic distribution of VBNP in cells: To determine the cell entry and selective uptake of VBNPs in cell cytoplasm, DF-1 chicken embryo fibroblast cells were infected with VBNPs and visualized by confocal microscopy post immunological staining with anti-NDV polyclonal chicken serum. The overlay shows VBNP uptake and subcellular localization only in the cytoplasm (green fluorescence) (Fig. 9), thus, confirming the ability of VBNPs to enter and get distributed in the cytoplasm of cells.

3.4.4 VBNPs are cytotoxic to tumor cells: To assess the cytotoxicity of VBNPs, 293T (human kidney epithelial cells) and different tumor cell lines; HT1080 (human fibrosarcoma), A549 (human non-small cell lung cancer), J3T (canine glioma) were infected with different concentrations of VBNPs (0.25 ng and 0.025 ng). The WST-1 assay indicated that VBNP and VBNP-VP3 iterated cells showed approximately 60% cytotoxicity in HT1080 and J3T (Fig.10A to B) tumor cell lines and also in 293T cells (Fig. 10C) but not in A549 cells (Fig. 10D). This indicated that VBNP and VBNP-VP3 were cytotoxic to tumor cells.

3.4.5 VBNP and VBNP- VP3 causes early apoptosis in tumor cell lines: To check for apoptotic ability of VBNPs, DF-1 cells treated with VBNP and VBNP-VP3 were processed for the detection of DNA fragmentation, using a DNA laddering kit. Intranucleosomal DNA fragmentation indicating apoptosis was observed in both the VBNP and VBNP-VP3 treated cells (Fig. 11).

Additionally, to understand the kinetics of apoptosis by VBNP and VBNP-VP3, we evaluated their apoptotic ability in different cell lines. VBNP treatment in DF-1 cells resulted in about 23% early apoptosis at 24 h post-infection and progressed to late apoptosis (about 42%) by 72 h (Fig. 12A). U87MG cells showed VBNP treatment induced early apoptosis whereas VBNP-VP3 treatment caused all the cells to die by 48 h (Fig. 12B). J3T cells had ~20% apoptosis when treated with VBNP whereas only about 14% early apoptotic cells were noticed with VBNP-VP3 treatment (Fig. 12C). Hence, it was concluded that both VBNP and VBNP-VP3 induced early apoptosis in tumor cell lines validating the potential oncolytic efficacy *in vivo* in tumor cells.

3.4.6 VBNPs elicit pro-inflammatory and chemotactic cytokines: To evaluate whether the VBNPs elicit an inflammatory and chemotactic response in tumor cells, HT1080 and U87MG cells were treated with VBNPs and assessed for pro-inflammatory and chemotactic cytokines. VBNP treatment in HT1080 cells induced a high IL-6 and IL-8 response and a lower MCP-1 response as compared to VBNP-VP3 and control cells whereas VBNP-VP3 treatment in U87MG cells was more significant than VBNP treatment and elicited a higher MCP-1 response compared to IL-6, IL-8 and RANTES (Fig. 13A to B). Other cytokines (IFN α , IL-10) and chemokines (IP-10, MIP-1 α) were not induced (data not shown) in any of these cells by different VBNPs.

3.4.7 VBNP dosage is insufficient to effect cures in fibrosarcoma and malignant glioma models: It has been shown earlier, that recombinant NDV strains like rBC-EGFP and rBC-Edit can effectively induce complete tumor regression in nude mice (Elankumaran et al., 2010). In order to determine the efficacy of VBNPs in effecting tumor regression, growth of implanted HT1080 or U87MG flank tumors in 6 week old Balb/c nude mice were followed in mock infected control groups or groups treated with VBNP, VBNP-VP3, and VBNP-VP3-fo on Days 0, 6, 18 and 28 (Table 1 and Table 2) at different doses.

There was no statistically significant reduction in tumor volumes in both tumor models when compared to the control group (Fig. 14A to B), although tumor growth was seen to be limited until the time of the last treatment (Day 28), suggesting that the doses tested were inefficient to effect a cure in these preclinical models. Further dose optimization studies are needed.

3.4.8 VBNP therapy prolonged survival in fibrosarcoma model: The Kaplan-Meier survival curves indicated that VBNP, VBNP-VP3 and VBNP-VP3-Fo had longer survival time compared to the control group (data not shown) in HT1080 tumor bearing mice. But, they were not statistically significant by the Kaplan-Meier survival analysis and the Log rank test (Fig. 15A).

3.4.9 VBNP treatment failed to prolong survival in malignant glioma: The Kaplan-Meier survival curve showed no difference in the VBNP-treated U87MG xenotransplant groups when compared to the mock-treated control group (Fig. 15B). The U87MG is generally a very aggressive tumor and at tested doses, the VBNPs were inefficient in prolonging the survival of these mice. Further, many mice had to be euthanized for humane reasons following the tumor policy at Virginia Polytechnic Institute and State University.

3.4.10 Histology of implanted tumors indicates VBNPs induced inflammatory response and necrosis: The H/E staining of histological tumor sections showed infiltration of megakaryocytes, mainly band neutrophils, in both HT1080 (Fig. 16A to D) and U87MG tumor implants (Fig. 17A to H) indicating a proinflammatory response. Similarly, the spleen sections showed extramedullary hematopoietic changes in U87MG induced tumors (Fig. 17A to H) compared to a normal spleen. Gross examination showed that mice with tumor sizes of > 0.5 cm in U87MG glioma had enlarged spleens without any correlation to the treatment. Tumor sections were scored based on the amount of visible microscopic necrosis. It was found that the VBNP-VP3 and the VBNP-VP3-fo groups had more evidence of necrosis compared to the other groups in both tumor types (Table 3), suggesting the ability of VBNPs to induce cell death.

3.5 Discussion

NDV based virus-like particles can be effective, very safe anti-tumor agents, although their potential has not been fully exploited. In this study, we have established a platform to generate oncolytic VBNPs by the directed expression of only three structural proteins of NDV. We have also demonstrated the flexibility of this approach to incorporate therapeutic transgenes by using a model proapoptotic protein (apoptin) and for ligand-directed targeting using folate conjugation on the surface. When examined by TEM, these VBNPs were reminiscent of authentic NDV virions in terms of size, particle morphology, and the fine structure of surface glycoprotein spikes. Further, the VBNPs contained the major surface glycoproteins and retained the biological properties such as fusion and apoptosis. We found that the VBNPs appeared to incorporate cellular proteins in the core by TEM which implied that the interactions between host and viral proteins involved in the biosynthesis of VBNPs probably reflected similar processes during virus assembly and budding as the authentic virions. Future studies are needed to identify the nature of cellular proteins incorporated by VBNPs.

We were successful in generating NDV VBNPs expressing apoptin (VP3) from chicken anemia virus and conjugating them with NHS-Folate. We were able to develop a proof of concept for the cytotoxicity of VBNP and VBNP-VP3 in tumor cells. We also found that VBNP and VBNP-VP3 were able to elicit IL-6, IL-8 and MCP-1 response in HT1080 and to some extent in U87MG tumor cell lines indicating that the VBNPs can elicit an effective antitumor response in the host. We have also successfully demonstrated that VBNPs were capable of entering and getting selectively distributed in the cytoplasm of tumor cells. We were able to confirm that VBNPs conjugated to folate specifically targeted tumor cells expressing FR on their surface.

From our *in vivo* studies, we found that VBNP, VBNP-VP3 and VBNP-VP3-fo were unsuccessful in effecting a complete tumor regression ($p>0.05$), although the tumor volumes over time indicated a controlled growth of tumor mass as long as the VBNPs were being administered. The antitumor immune response of the VBNPs, VBNP-VP3 and the folate conjugated VBNP has to be further investigated in immunocompetent and nude mouse tumor models for dose optimization (the frequency and quantity of VBNP).

In summary, we have established a VBNP construction system for NDV and demonstrated that they are capable of inducing apoptosis in tumor cells. We were able to surface modify the VBNPs by incorporating foreign genes/proteins using only the structural core M protein and CT and TM of HN protein, thus increasing their capacity to carry multiple or long transgenes. Our results present a novel and flexible platform for developing NDV VBNP as a powerful vaccine vector or cancer therapeutic agent. Besides, the VBNPs could be effectively used for studying the basic paramyxoviral assembly and budding processes.

3.6 Tables

Treatment group	Number of mice/group	Mean tumor volume (cm ³)	
		Day 0 (start of Rx)	Day 28 (end of Rx)
PBS	4	0.2275	1.2632
VBNP	5	0.1326	1.7635
VBNP 1/10	6	0.1861	0.9312
VP3	6	0.1843	0.8811
VP3 1/10	6	0.0720	0.9300
VP3-FO	6	0.0950	1.0762
VP3-FO 1/10	6	0.1381	1.0939

Table 1. Mean tumor volume of xenotransplanted HT1080 fibrosarcoma in nude mice: Mice injected with HT1080 tumor cells were subjected to multiple VBNP, VBNP-VP3, VBNP-VP3-Fo or PBS treatments at various concentrations as described in material and methods. The mean tumor volumes at the beginning of treatment (Day 0) and the last day of treatment (Day 28) are indicated for each group. Rx: treatment.

Treatment group	Number of mice/group	Mean tumor volume (cm ³)	
		Day 0 (start of Rx)	Day 28 (end of Rx)
PBS	4	0.0905	0.3912
VBNP	5	0.1008	1.0727
VBNP 1/10	4	0.0893	0.4832
VP3	5	0.0743	1.1790
VP3 1/10	5	0.0836	1.2367
VP3-FO	3	0.0833	1.7250
VP3-FO 1/10	5	0.0808	0.7332

Table 2. Mean tumor volume of xenotransplanted U87MG malignant glioma in nude mice: Mice implanted with U87MG tumor cells were subjected to multiple VBNP, VBNP-VP3, VBNP-VP3-Fo or PBS treatments at various concentrations as described in materials and methods. The mean tumor volumes at the beginning of treatment (Day 0) and the last day of treatment (Day 28) are indicated for each group. Rx: treatment.

Treatment groups	Mean histological lesion scores (percent necrosis)	
	HT1080 implants	U87MG implants
VBNP	20	45
VBNP-VP3	40	90
VBNP-VP3-fo	70	85
Mock-infected	20	60

Table 3. Histological lesion scores of xenotransplanted tumors in nude mice: H&E staining of paraffin-embedded tissue sections of tumor mass from each group of treated and control mice from HT1080 and U87MG tumors were scored based on the amount of visible microscopic necrosis evident in the tissue sections. The data shows mean percent tumor necrosis (n=5) for each group.

3.7 Figures

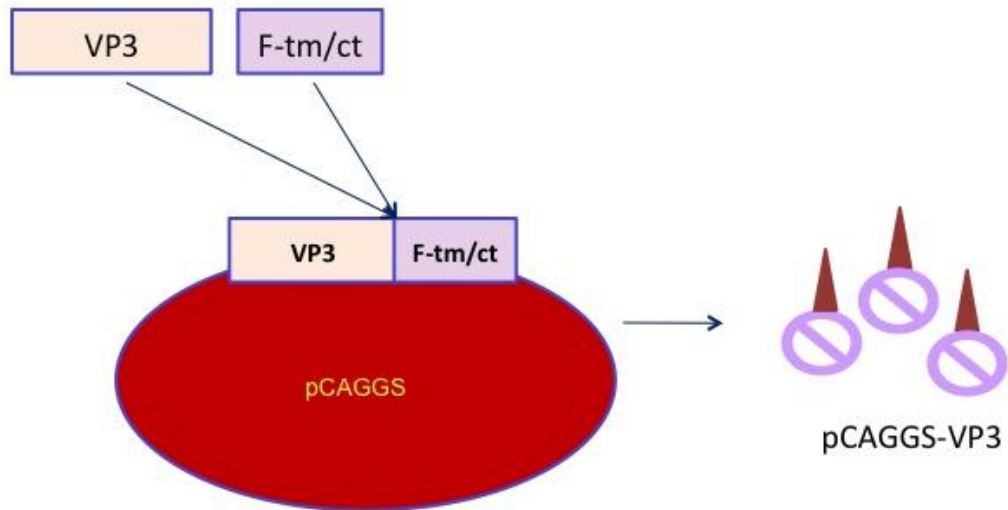


FIGURE 1. A schematic representation of construction of VBNP-VP3 plasmid: The VP3, apoptin gene from chicken anemia virus was fused with the transmembrane (TM) and cytoplasmic tail (CT) sequences from the NDV-F gene and the resulting VP3/F-tm/ct fusion gene was cloned into pCAGGS expression plasmid to obtain the pCAGGS-VP3 plasmid.

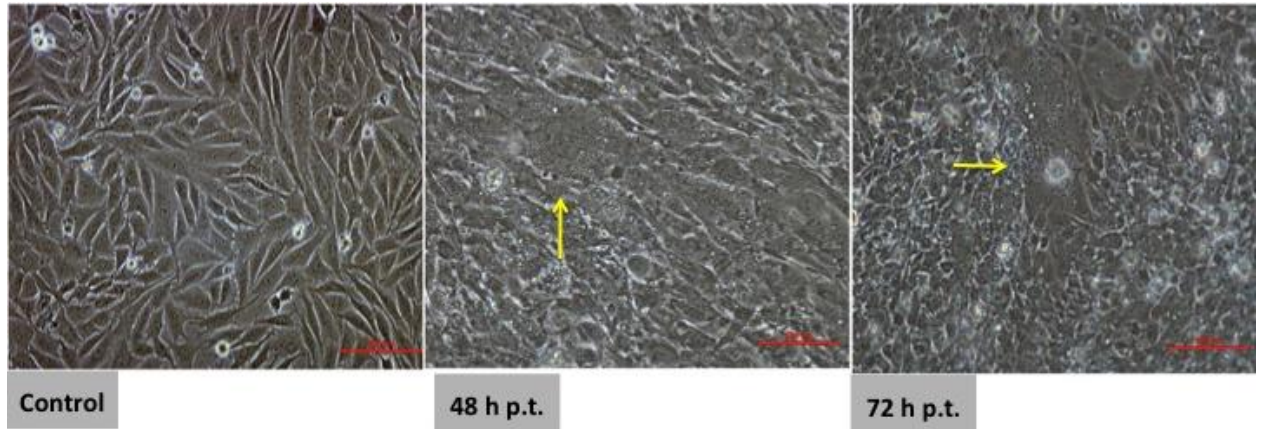


FIGURE 2. Fusogenicity of VBNPs in DF-1 cells: DF-1 cells were transfected with pCAGGS- HN, -M and -F plasmids as described in materials and methods (section 3.3) and syncytia were observed at 48 and 72 h post-transfection by phase contrast microscopy (Nikon Eclipse) at 20 x magnification.

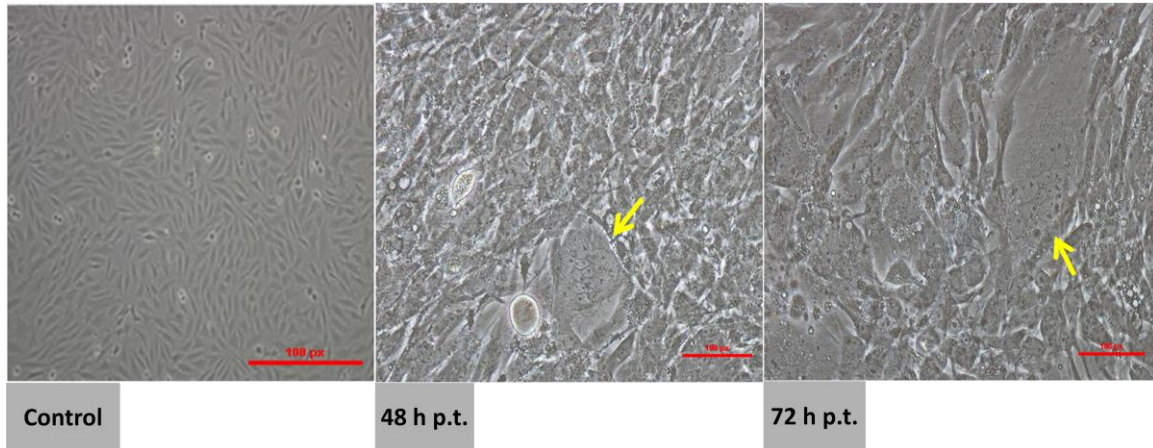


FIGURE 3. Fusogenicity of VBNP-VP3 in DF-1 cells: DF-1 cells were transfected with pCAGGS -HN, -M, -F and -VP3 plasmids as described in materials and methods (section 3.3) and syncytia were observed at 48 and 72 h post-transfection by phase contrast microscopy (Nikon Eclipse) at 20 x magnification.

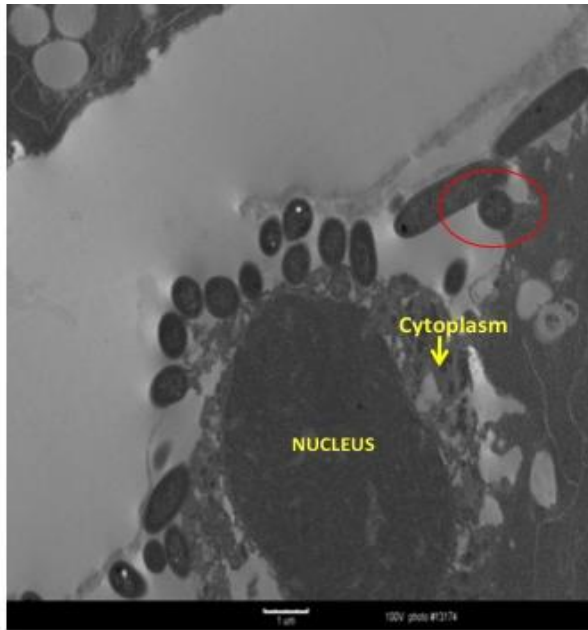
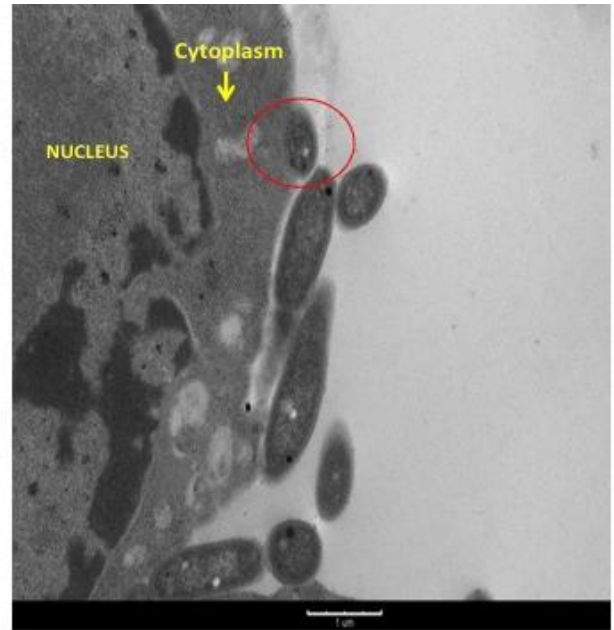
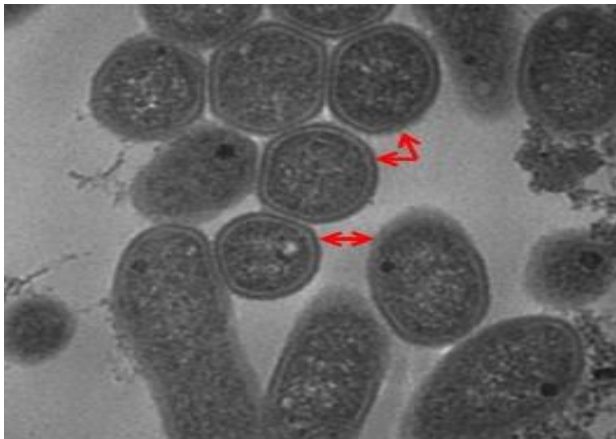
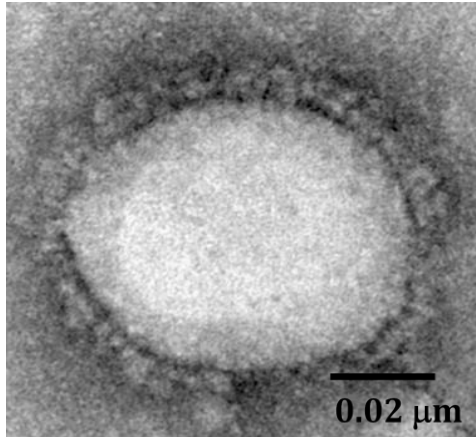
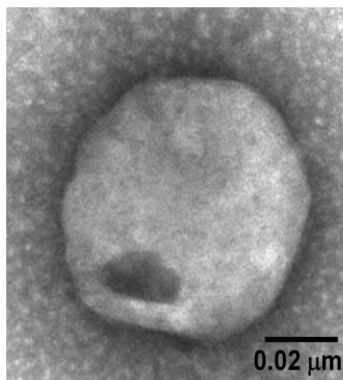
A**B****C**

FIGURE 4. Electron micrograph of VBNP budding and release in 293T cells: The assembly of VBNPs in the cytoplasm of the cells is evident (A and B) and these particles bud out from the cell cytoplasm acquiring the cellular membrane revealing structural similarities to authentic NDV virus particles. The particles were pleomorphic in shape and the particle sizes ranged from 50- 300nm (C).

A.



B.



C.

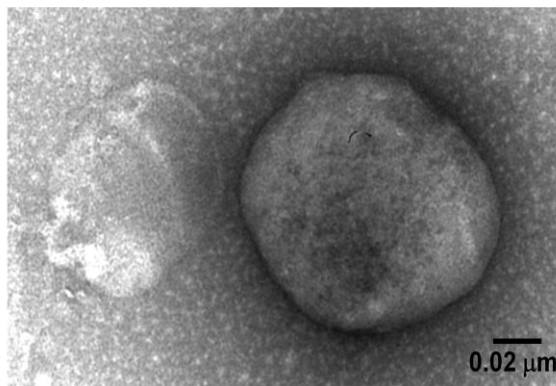


FIGURE 5. Transmission electron microscopy (TEM) of negatively stained NDV and NDV-VBNPs: The NDV (A) or VBNPs (B and C) were visualized after adsorption on nickel grids and negative staining with 2% phosphotungstic acid. The particles were examined at 55,000 x magnification under a Phillips 400 TEM. Note prominent and fine glycoprotein spikes on the surface of the virus (A) and VBNPs (B and C) giving them a rugged end look. Both authentic virus particles and VBNPs show evidence of cellular proteins in the core. Note the absence of nucleocapsid in the VBNPs.

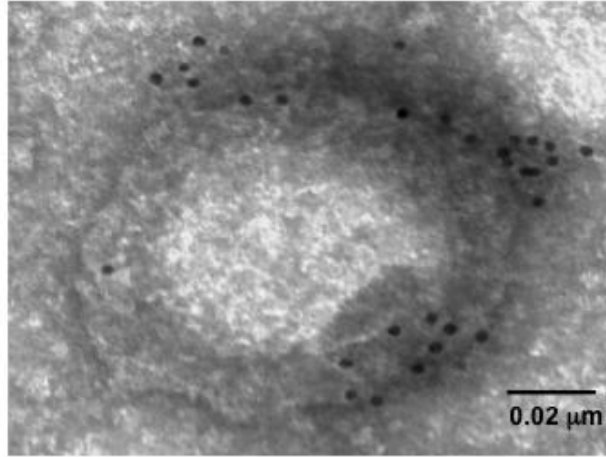


FIGURE 6. Immunogold transmission electron microscopy (iTEM) of VBNP: VBNPs were immunogold labeled using chicken anti-NDV serum bound to 12 nm colloidal gold donkey anti-chicken IgY antibody, followed by negative staining and visualized under a Philips 400 TEM at a magnification of 55,000 x. Note specific immunogold labeling of the surface projections on the NDV-VBNPs.

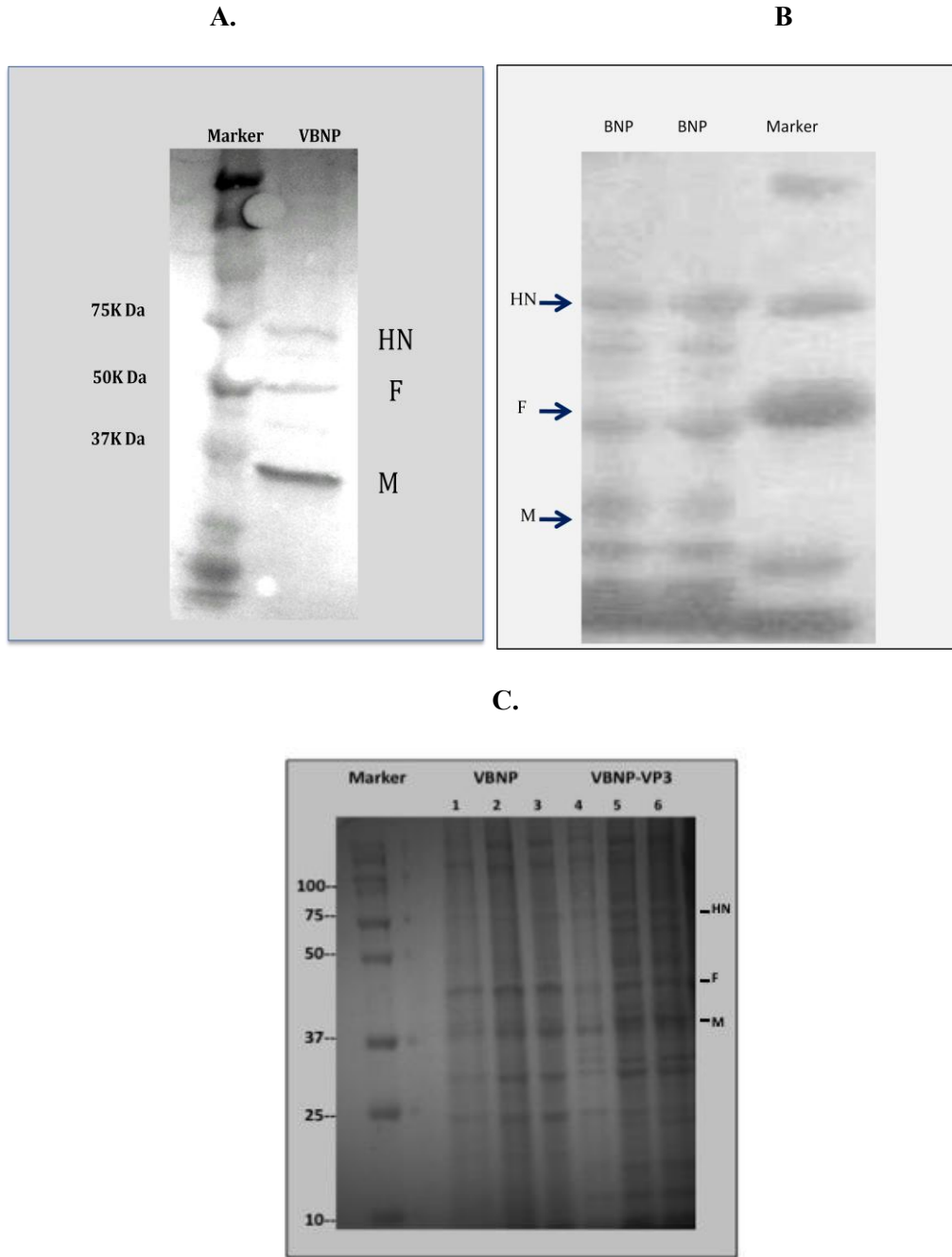


FIGURE 7. VBNPs incorporated NDV structural proteins and cellular proteins:

NDV glycoproteins that were employed to generate VBNPs (HN, M and F proteins) were detected by SDS-PAGE and confirmed on Immunoblot using anti-NDV chicken serum (A, B). Coomassie blue stained gels revealed the presence of cellular proteins in purified VBNPs in addition to viral proteins (C).

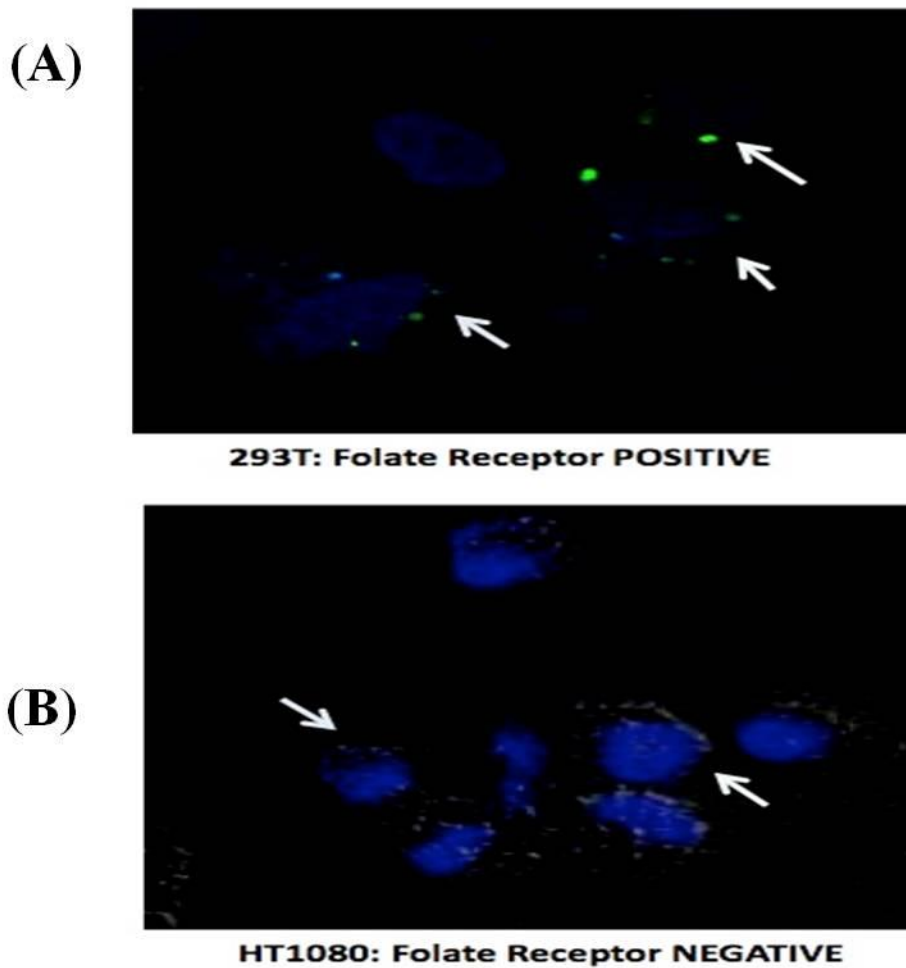


FIGURE 8. VBNP uptake in cells expressing folate receptor: Folate-FITC conjugated VBNP uptake (green) was observed only in Folate receptor (FR) positive cells (293T) (A) and undetectable in FR negative cells (B) by confocal microscopy. Cell nuclei were stained with DAPI (blue).

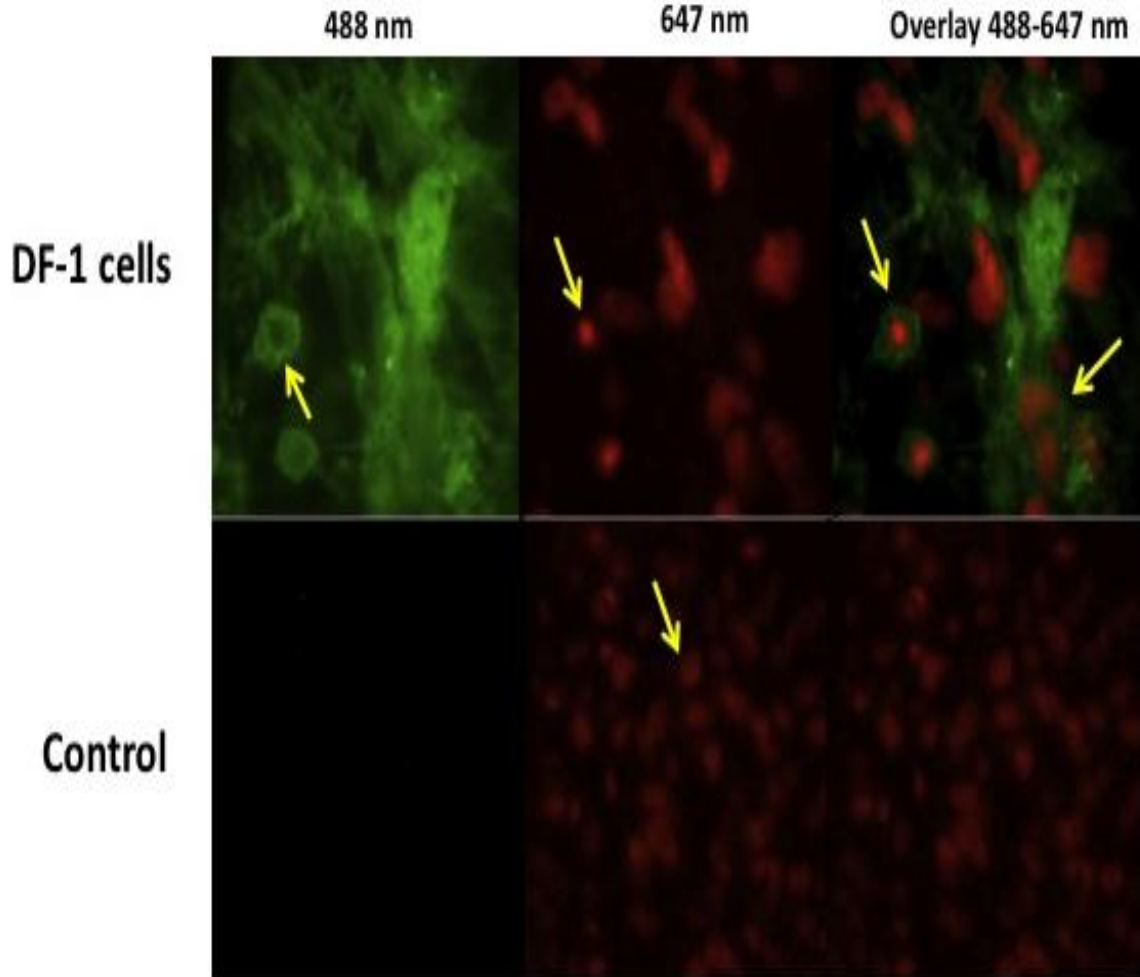
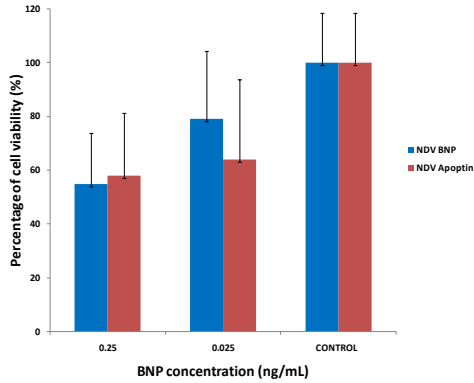
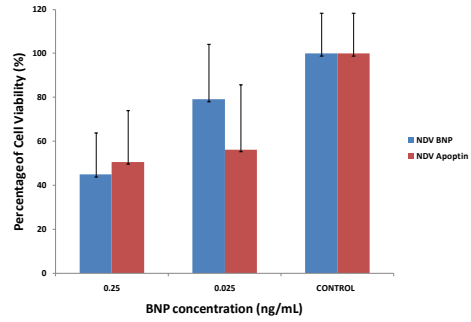


FIGURE 9. Cell entry and cytoplasmic distribution of NDV VBNPs: Treatment of DF-1 cells with VBNPs revealed selective uptake of VBNPs in cell cytoplasm by immunofluorescence, after indirect immunofluorescence staining with polyclonal anti-NDV chicken serum. The overlay shows VBNP uptake only in the cytoplasm (green) at 488 nm wavelength in DF-1 infected cells, thus, confirming the ability of VBNPs to enter and get distributed only in the cytoplasm of cells. Cell nuclei were stained with a nuclear stain Syto60 (red).

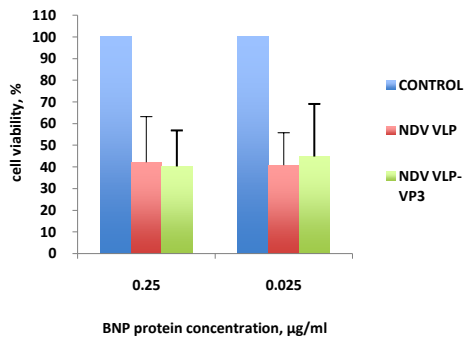
A.



B.



C.



D.

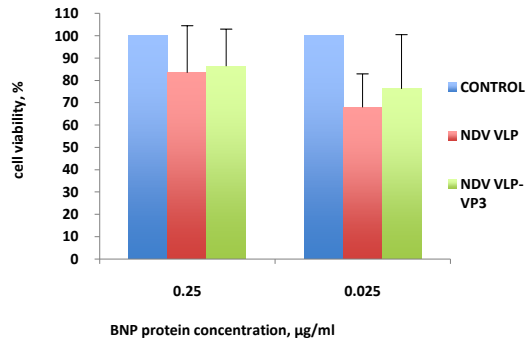


FIGURE 10. VBPNs induce cytotoxicity in treated transformed and tumor cells: J3T

(A) and HT1080 (B), 293T (C), and A549 (D) cells were treated with VBPN and VBPN-VP3 in a 96 well plate (Corning). After 72 h incubation, 10 µl WST-1 reagent was added to each well and incubated for additional 2 h. The resulting number of viable cells was calculated by measuring the absorbance produced in each well compared to mock-infected control cells. VBPN-VP3 showed higher cytotoxicity than VBPN in treated cells. VBPN-treated 293T cells showed significant cytotoxicity compared to the control but no appreciable cytotoxicity was detected in A549 cells.

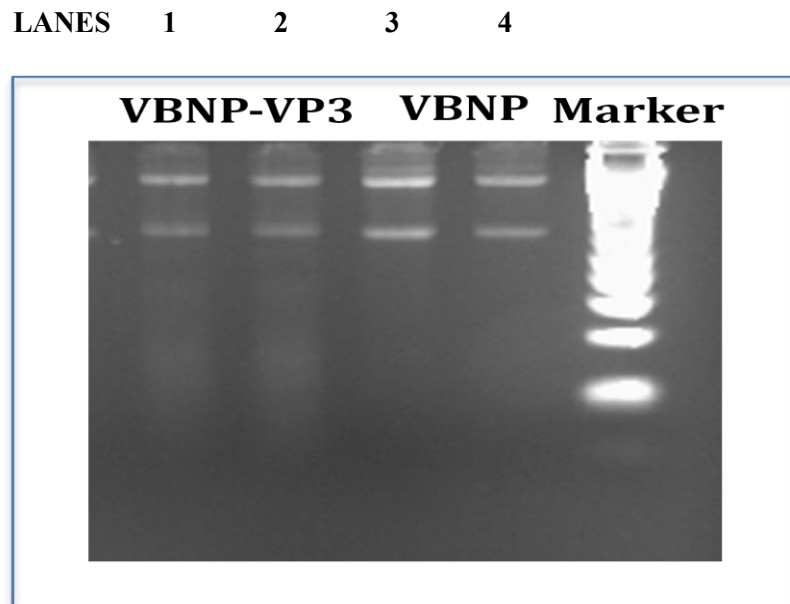
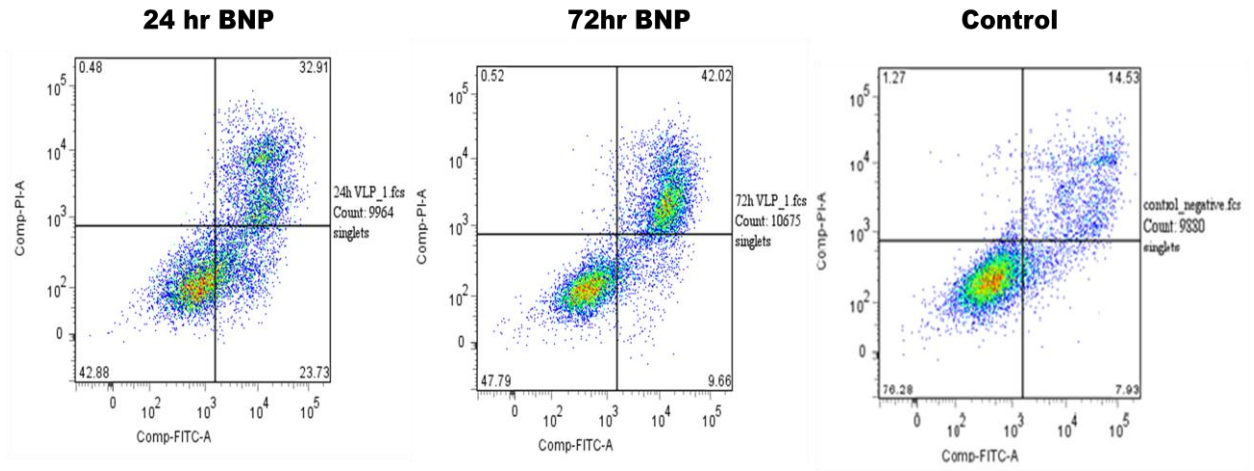


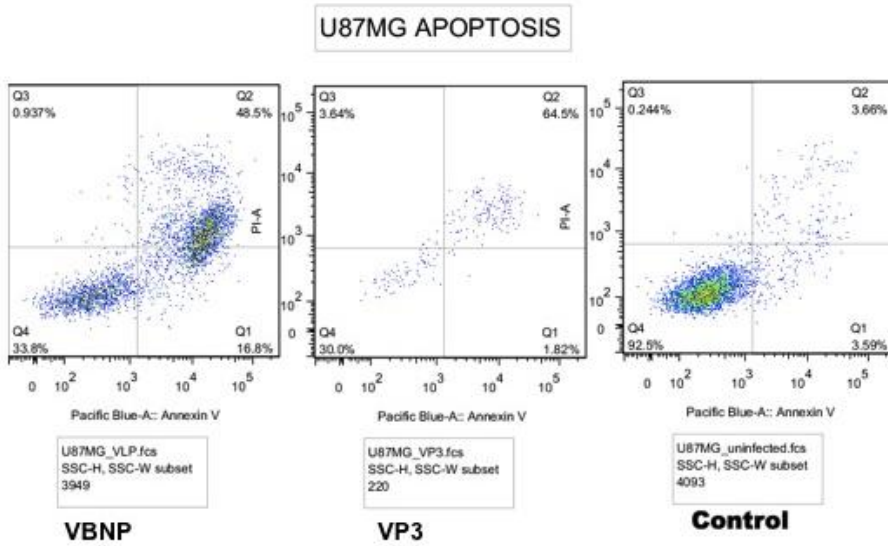
FIGURE 11: VBNP treated cells undergo intranucleosomal DNA fragmentation.

VBNP-VP3 (lanes 1, 2) and VBNP (lanes 3, 4) treated cells were processed using Apoptosis DNA Laddering kit (Roche) as described in materials methods (section 3.3). Nuclear fragmentation was observed as formation of laddering DNA bands and smearing in VBNP and VBNP-VP3 lanes.

A.



B.



C.

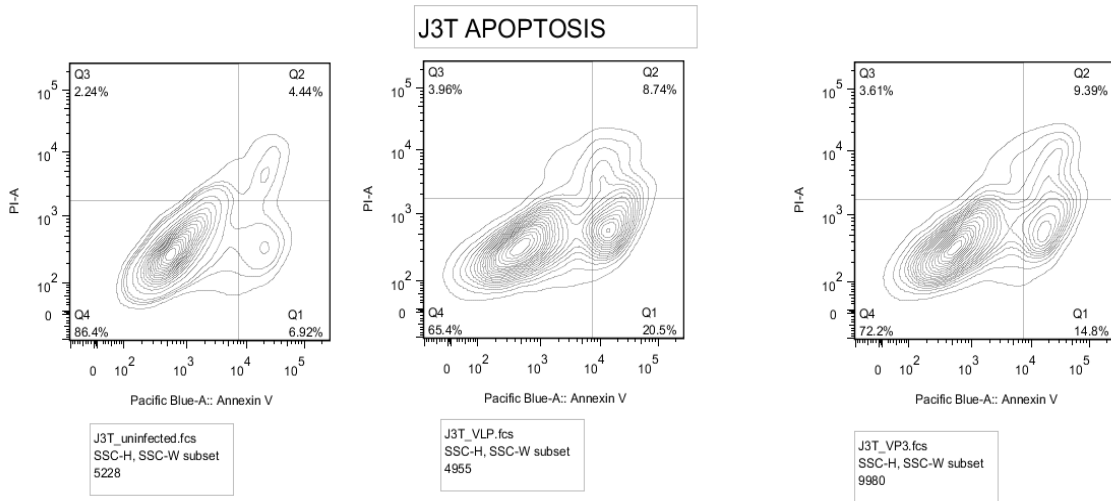
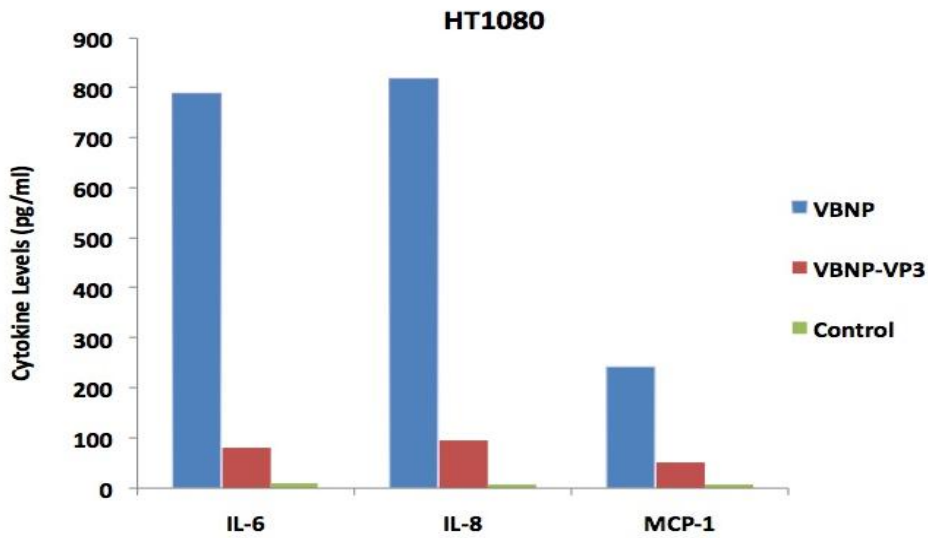


FIGURE 12. NDV VBNPs induce early apoptosis: Flow cytometric analysis of VBNP and VBNP-VP3 treated cells after staining with propidium iodide and FITC-Annexin V (U87MG and J3T) or PacBlue-Annexin V (Df-1 cells) to detect dead and apoptotic cells. DF-1 cells showed percent early apoptotic cells (lower right quadrant) at 24 h post-infection and late apoptosis (upper right quadrant) by 72 h were indicated (A). VBNPs elicited early apoptosis in U87MG cells whereas VBNP-VP3 induced all the cells to die by 48 h (B). Whereas J3T cells had ~20% apoptosis when treated with VBNP but only about 14% early apoptotic cells (lower right quadrant) were noticed with VBNP-VP3 treatment (C).

A.



B.

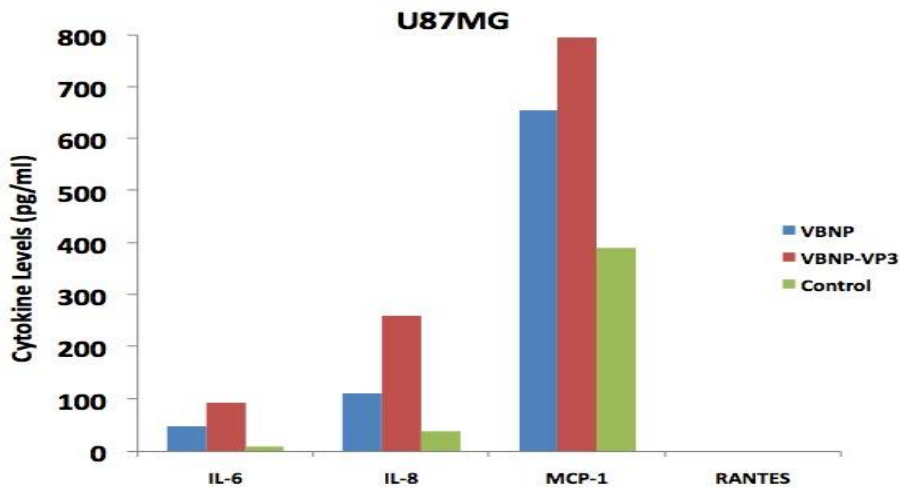
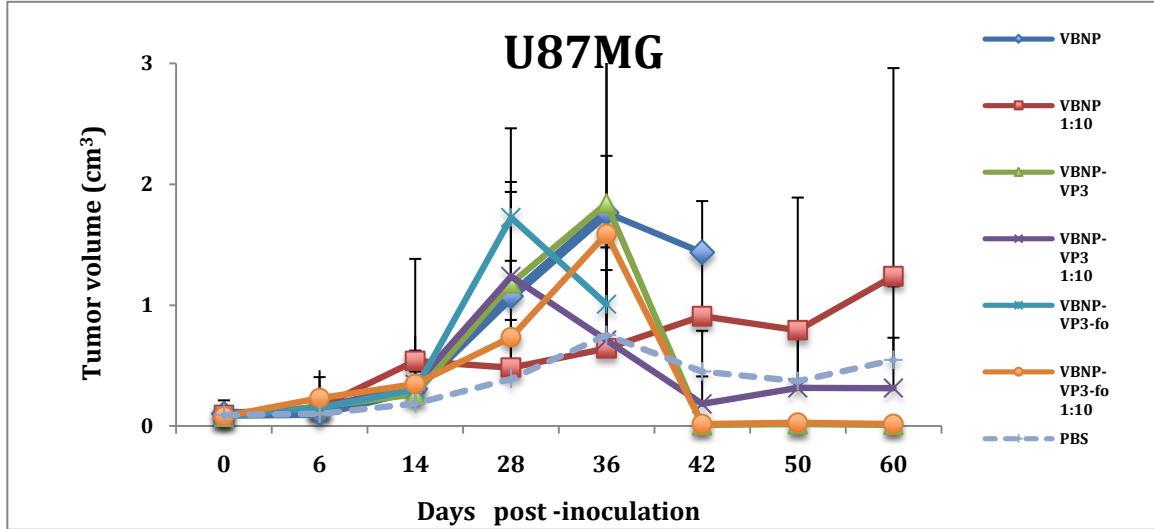


FIGURE 13. VBNPs induce proinflammatory cytokines and chemokines in tumor

cells: HT1080 and U87MG cells were treated with VBNPs and VBNP-VP3 and analyzed for proinflammatory and chemokine response by cytometric bead assay. In HT1080 cells, VBNPs elicited a comparatively higher IL-6 and IL-8 response to VBNP-VP3 and control groups (A) whereas MCP-1 and to some extent IL-8 response was higher in VBNP-VP3 infected U87MG cells (B).

A.



B.

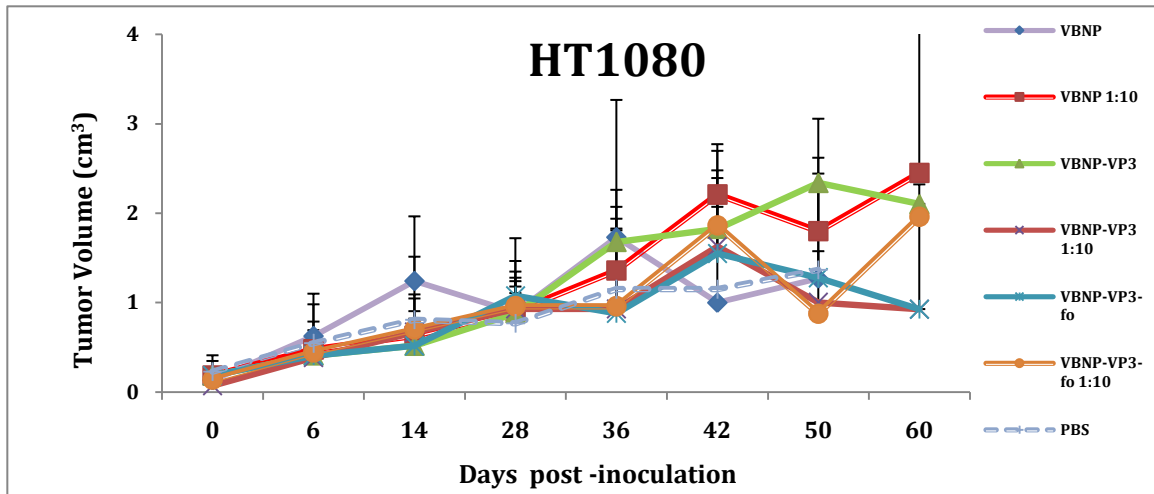
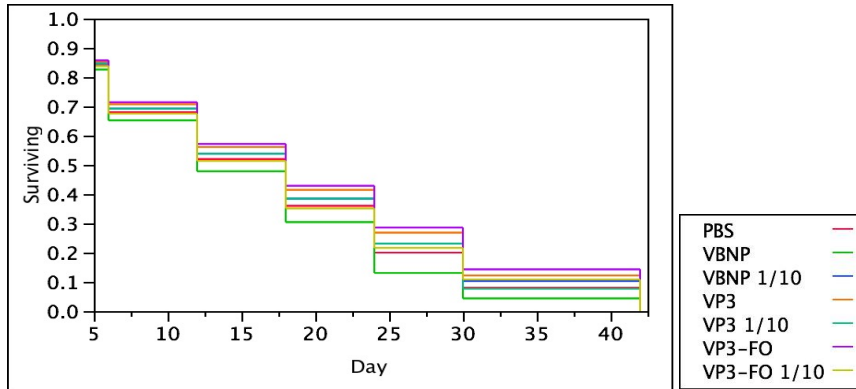


FIGURE 14. Mean tumor volume in VBNP treated xenotransplanted tumors: The tumor dimensions were observed every other day and the tumor size was calculated for tumor volume and average of each group was plotted in a graph over a period of time in days post treatment for each group. Although no statistical significance was observed for tumor regression in both HT1080 (A) and U87MG (B) tumor implants, graphical representation of the tumor volume indicated a controlled growth of tumor mass until end of treatment (Day 28).

A.



B.

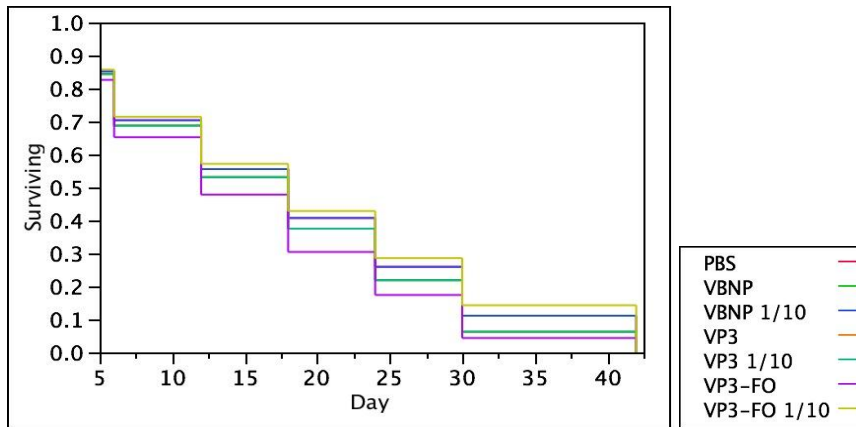


FIGURE 15. VBNP treatment in both HT1080 and U87MG failed to prolong the survival times in tumor bearing mice: Surviving mice from each group was plotted to determine the percent survival for each group in both HT1080 (A) and U87MG (B) using Kaplan Meier method. No statistical significance was observed.

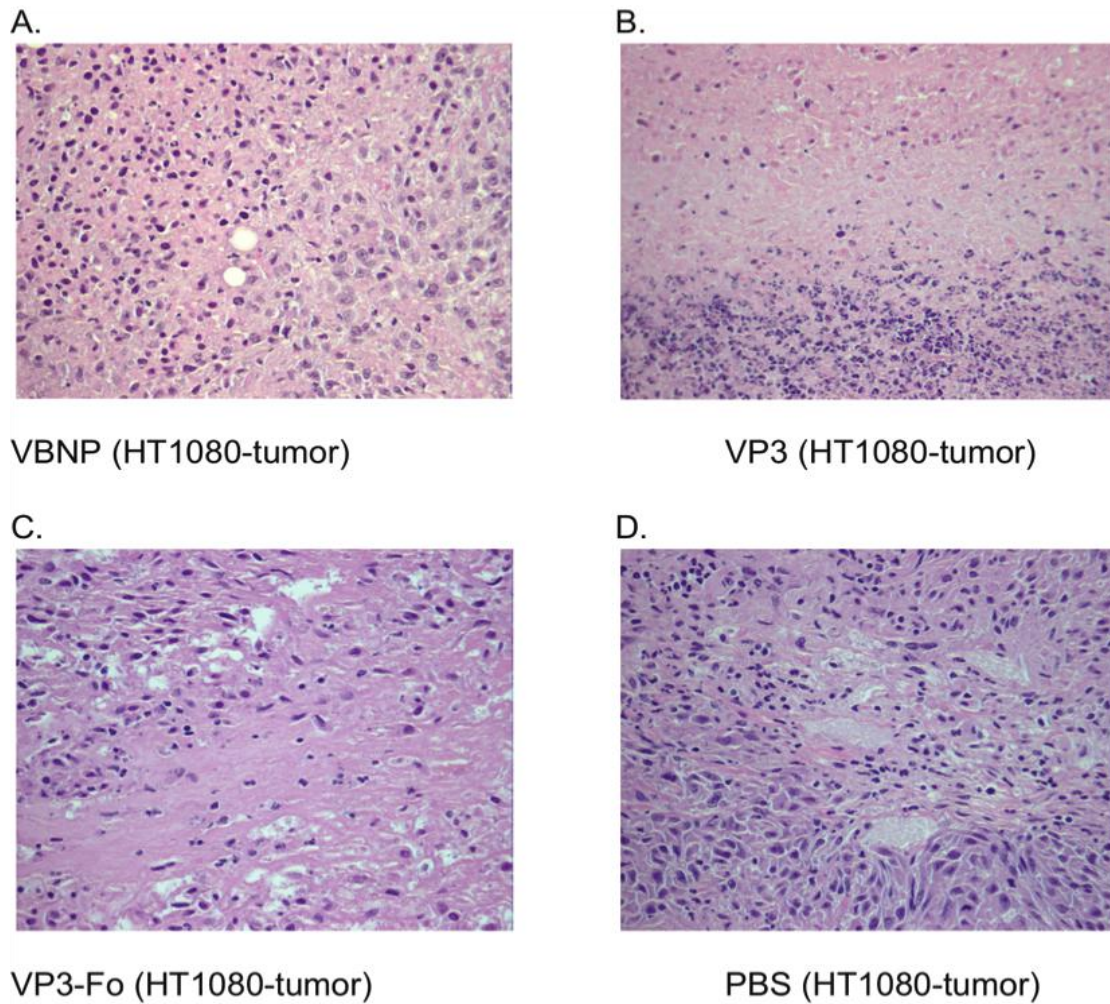


FIGURE 16. VBNP therapy induced a proinflammatory response and cell death in implanted tumors: The H&E stained histological sections of HT1080 implants showed infiltration of megakaryocytes, mainly band neutrophils. The pink streaking indicates areas of necrosis containing fragmented neutrophilic infiltration. VBNP (A), VBNP-VP3 (B), VBNP-VP3-Fo (C) and mock-infected control (D).

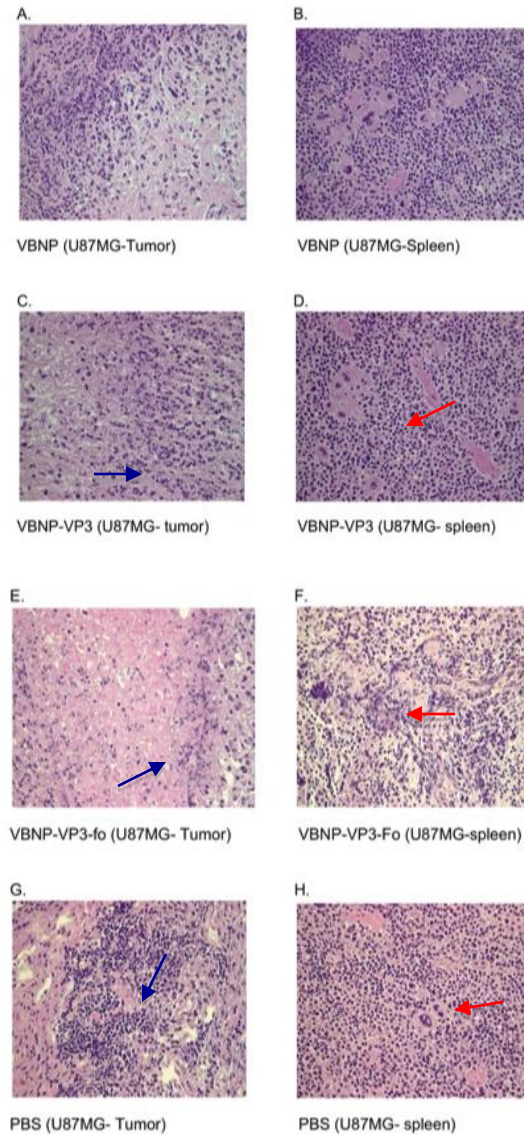


FIGURE 17. VBNP therapy kills tumor cells by necrosis and causes splenomegaly in U87MG xenotransplanted mice: Representative H&E stained histological tumor sections showed infiltration of megakaryocytes, mainly band neutrophils as indicated with arrows (blue), irrespective of treatment. The pink streaking indicates areas of necrosis containing fragmented neutrophilic infiltration (A, C, E). Gross examination showed splenomegaly. The spleen sections showed extramedullary hematopoietic changes (red arrows) in U87MG induced tumors (B, D, F).

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3.9 Acknowledgements

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Chapter 4: Surface Modification of Newcastle disease Virus-Based Nanoparticles with Nipah Virus Glycoproteins

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4.1 Abstract

Extensive advancements have been made in the past few years towards understanding both unique and shared processes between paramyxoviruses during virus particle formation. Nipah (NiV) and Hendra viruses are novel viruses which fall under the genus henipavirus, in the family *Paramyxoviridae*. They are responsible for causing deadly infections in humans and domestic animals with severe vasculitis and encephalitis. There are presently no successful treatments or vaccines approved for henipavirus infections. NiV is a single-stranded, negative-sense RNA virus that encodes six structural proteins including two envelope glycoproteins: the G and F protein. G protein is responsible for binding to the cellular receptors, ephrin B2 and the F protein mediates membrane fusion. It has been known that high expression of ephrin-B is associated with increased tumor growth, tumorigenicity and metastasis. For paramyxoviruses, viral M proteins are the organizers of this assembly process and are highly abundant that bind directly to cellular membranes and occupy a central position allowing interaction both with viral RNP cores and also with viral glycoproteins via the cytoplasmic tail. Therefore, we hypothesized that the core structural M protein of NDV independently could incorporate surface glycoproteins from other paramyxoviruses without the help of transmembrane cytoplasmic tail sequences of

NDV glycoproteins. We tested this hypothesis by co-expressing NDV M gene with NiV F and G glycoproteins to form virus-based nanoparticles (NiV-ndBNP) in 293T cells and studied the morphology and biological characteristics of authentic NiV virus-like particles and NiV-ndBNPs. Our results demonstrated that NDV M successfully incorporated NiV glycoproteins on the envelope, and the resulting NiV-ndBNPs were fusogenic, and were reminiscent of authentic NDV/NiV virions. These novel NiV-ndBNPs could be developed for efficient targeting to ephrinB2/B3 receptor bearing tumor cell types and as vaccines/diagnostics for NiV.

4.2. Introduction

Nipah and Hendra viruses are novel viruses which fall under the genus henipavirus, in the family *Paramyxoviridae*. They are responsible for causing deadly infections in humans and domestic animals with severe vasculitis and encephalitis resulting in about 40% mortality rate (Bishop and Broder, 2008). Fruit bats, such as flying foxes are the natural hosts for these zoonotic viruses. Pigs are intermediary hosts for Nipah virus (NiV) infections and horses in the case of hendra virus (Eaton et al., 2006). There are presently no successful treatments or vaccines approved for henipavirus infections. Outbreaks of hendravirus have potential agricultural significance; NiV epidemic in Malaysia was responsible for culling 1.1 million pigs after which the epidemic was under control (Mohd Nor et al., 2000).

NiV is a single-stranded, negative-sense RNA virus and consists of six genes (N-P-M-F-G-L) flanked by a 3' leader and 5' trailer region with a genome length of 18246 nucleotide (nt) in Malaysian isolate or 18252 nt in Bangladesh isolate (Mungall et al., 2007) (Fig. 1). NiV has a pleomorphic structure ranging from 50 nm to greater than 600 nm in diameter (Hyatt et al., 2001). It has six transcription units that encode six structural proteins including two envelope glycoproteins: the G and F protein. G protein is responsible for binding to the cellular receptors, ephrin B2 and B3 (Bonaparte et al., 2005; Negrete et al., 2005) and the F protein mediates membrane fusion (Bossart et al., 2002). M protein is located below and interacts with both the glycoproteins and the ribonucleoprotein (RNP) complex (Lamb and Parks, 2007; Schmitt and Lamb, 2004). Like other paramyxoviruses, the P gene of NiV expresses four proteins, namely P, V, W and C (Harcourt et al., 2000; Wang et al., 2001).

For some paramyxoviruses, such as Sendai virus, NDV and MuV, attachment is mediated by HN proteins that bind to sialic acid receptors (Lamb et al., 2006). Other paramyxovirus attachment proteins mediate binding to protein receptors like the H protein of MeV and the G

proteins of the henipaviruses (Russell and Luque, 2006). The receptor for the attachment glycoprotein (G) of NiV is ephrin B2/B3 (Ciancanelli and Basler, 2006; Wild, 2009). The Ephrin B2/B3, binding site for NiV G resides in a similar position as HN protein sialic acid binding sites (Xu et al., 2008). NiV interacts with ephrin-B2 and -B3 via an overlapping site on the virus G attachment protein (Negrete et al., 2006). NiV forms particles by budding from the cellular membranes.

Ephrins have been recognized to be differentially expressed in various human tumors such as malignant melanoma, neuroblastoma and cancers of the prostate, breast, lungs, esophagus, gastrum and colorectum (Kiyokawa et al., 1994). Evidence has shown that ephrin-B signaling is associated with cancer progression (Campbell and Robbins, 2008; Merlos-Suarez and Batlle, 2008; Pasquale, 2008). An increased activity and expression of ephrin-B receptors have been interrelated with the growth of solid tumors (Alam et al., 2009; Heroult et al., 2006; Irie et al., 2008; Merlos-Suarez and Batlle, 2008). Besides, high expression of ephrin-B is associated with increased tumor growth, tumorigenicity and metastasis (Kiyokawa et al., 1994; Nakada et al., 2009; Tang et al., 1999).

Paramyxovirus assembly can be viewed as a three-part process. First the genomic RNA associate with NP subunits to form the helical nucleocapsid structure, and then the P and L proteins are added (Harrison et al., 2010) to form the RNP. The membrane proteins (HN, F, and M) then accumulate at the plasma membrane. The nucleocapsid is enveloped during the budding process and progeny virions are produced. Viral M proteins are the organizers of this assembly process. These highly abundant viral proteins bind directly to cellular membranes and occupy a central position that allows interaction both with viral RNP cores and also with viral glycoproteins via the cytoplasmic tail (CT). For paramyxoviruses, viral M proteins are key drivers of virus assembly and budding (Harrison et al, 2010). At present, the protein-protein

interaction that regulates the incorporation of paramyxovirus glycoproteins into virions and the detailed sequence specificity involved in these processes are not known.

The M protein is one of the abundant proteins in the virion and plays a multifaceted role in paramyxovirus replication. Expression of NiV M protein alone leads to the budding of VLP's. A late domain sequence (YMYL) is important for this property (Ciancanelli and Basler, 2006; Wild, 2009). Similarly, M protein of NDV occupies a central position that allows interaction both with viral RNP cores and also with viral glycoproteins via the CTs incorporating them into viral particles (Harrison et al., 2010). Recently, it has been shown that NDV structural core proteins, NP and M protein were able to incorporate the ectodomain of respiratory syncytial virus G protein fused to the CT and transmembrane (TM) sequences of the NDV HN protein (McGinnes et al, 2010). We have also incorporated the chicken anemia virus VP3 protein on the surface of NDV in addition to the NDV HN and F proteins but used only the M protein of NDV for the structure (Chapter 3).

We hypothesize based on the above findings that the core structural M protein of NDV independently could incorporate surface glycoproteins from other paramyxoviruses without the help of TM and CT sequences of NDV glycoproteins. As a proof of concept, we tested this hypothesis by co-expressing NDV M gene with NiV F and G glycoproteins (NiV-ndBNP) in 293T cells. The morphology and biological characteristics of authentic NiV virus-like particles and NiV-ndBNPs were compared. Our results demonstrated that NDV M successfully incorporated NiV glycoproteins on the envelope, and the resulting NiV-ndBNPs were fusogenic, and were reminiscent of authentic NDV/NiV virions. These novel NiV-ndBNPs could be exploited for efficient targeting to ephrinB2/B3 receptor bearing tumor cell types and as vaccines/diagnostics for NiV.

4.3. Materials and Methods

4.3.1. Cells: 293T human embryonic kidney cells and DF1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco Inc.) supplemented with penicillin-streptomycin (Gibco Inc.) and 10% fetal calf serum (FCS) (Fisher Scientific).

4.3.2 Plasmids and antibodies: NDV cDNA sequences encoding the M protein were cloned into the expression vector pCAGGS to generate pCAGGS-M. NiV cDNA sequences encoding M, F and G proteins were cloned into the expression vector pCMV3Tag1 and pCAGGS to generate pPCMV-3Tag1-M (Wang et al., 2010), pCAGGS-F and pCAGGS-G plasmids, respectively. The plasmid pCMV-3tag1-M was a kind gift from Dr. Benhur Lee from University of California, Los Angeles (UCLA). The plasmids pCAGGS-F and pCAGGS-G were obtained from Dr. Paul Rota, Centers for Disease Control, Atlanta. Chicken polyclonal anti-NDV serum (SPAFAS) was used to probe for NDV structural proteins. Monospecific serum against NiV F and G structural proteins, raised in rabbits were gifts from Dr. Benhur Lee, UCLA.

4.3.3 Generation of NiV-BNP and NiV-ndBNP: Transfections of subconfluent 293T cells were accomplished using Lipofectamine 2000 (Invitrogen Inc.). The following amounts of plasmid DNA were used per 150-mm dish (Corning Inc.): 10µg each of pPCMV-3Tag1-M, pCAGGS-F NiV, and pCAGGS-G NiV for NiV VBNP and pCAGGS-M NDV, pCAGGS-F NiV, and pCAGGS-G NiV for NiV-ndBNP. For each transfection, a mixture of each of the plasmid DNA with 2 mL OptiMEM medium (Gibco Inc.) and 10 µl of Plus reagent (Invitrogen Inc.) in tube A and 2 mL OptiMEM medium with 20 µl Lipofectamine 2000 (Invitrogen Inc.) in tube B were incubated at room temperature for 20 min. Tube A and B were mixed and further incubated

for 20 min at room temperature. The mixture was then added to each plate after washing it 3 times with phosphate buffered saline (PBS), pH 7.4 and further incubated at 37°C, 5% CO₂ for 4-6 h. DMEM with 2% FCS and Penicillin-Streptomycin (Gibco Inc.) was then added to make up the volume to 20 mL. At 72 h post-transfection, cells were harvested with a cell scraper (Corning Inc.) and clarified at 5000g for 30 min.

4.3.4 NiV-BNP and NiV-ndBNP purification: The clarified supernatants were pelleted by centrifugation in a SW 28 rotor in a Beckman ultracentrifuge (Beckman Inc., USA) at 25,000 rpm for 2 h at 4°C. The pellet was re-suspended in TNE buffer (25 mM Tris- HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) (Pantua et al., 2006) and overlaid on top of a step gradient consisting of 5 ml 20% and 3 ml 55% sucrose solutions (g/ml) in Ca/Mg free PBS, pH 7.4, and centrifuged using a SW 41 rotor at 22,000 rpm for 2 h at 4°C. The band at the interface (containing concentrated particles) was collected and was diluted in TNE buffer and centrifuged in a SW 28 rotor at 25,000 rpm for 2 h at 4°C for concentrating the NiV-BNP and NiV-ndBNP. The pellet was then re-suspended in 500 µl of TNE buffer and stored at 4°C until use. The purified BNPs were further filtered through Amicon Ultra-15 centrifugal filter (Millipore Inc.) at 5000g for 15 min. The retentate was collected containing the filtered and concentrated BNPs and was stored at 4°C.

4.3.5 Transmission Electron microscopy (TEM): The purified NiV-ndBNPs were adsorbed to formvar carbon-coated 200 mesh nickel grids (Electron Microscopy systems). The grids were then washed in PBS, pH 7.4 and fixed with 2% paraformaldehyde (10 min) and extensively washed with PBS, pH 7.4 again before negatively staining with 1% phosphotungstic acid. The morphologic characteristics were examined using a Philips 420 transmission electron

microscope at a magnification of 55,000 x, operating at 80 kV.

4.3.6 Immunogold Transmission Electron Microscopy: NiV-ndBNP suspension (10 μ l) was layered on carbon coated 200 mesh gold grids (Electron Microscopy systems) and incubated for 1h at 4^oC, after which the remaining suspension was removed. The grids were blocked with PBS, pH 7.4 containing 1% BSA. Adsorbed NiV-ndBNPs were then stained with a mixture of both anti-F and anti-G rabbit polyclonal antibodies against NiV at a dilution of 1:25. Bound antibody was detected with 12 nm colloidal gold, goat anti-rabbit antibodies (Jackson Immunoresearch), followed by negative staining and analysis under Philips TEM 400 transmission electron microscope at a magnification of 55,000 x, operating at 80 kV.

4.3.7 ImmunoBlot: The gradient purified NiV-BNPs and NiV-ndBNPs were subjected to immunoblotting using standard procedures. The proteins were separated in a 4-20% sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P (Millipore) membranes. The membranes were blocked overnight in 5% skim milk and 0.05% Tween 20 solution at 4^oC and washed in PBS, pH 7.4 with 0.05% Tween 20 (PBST) and incubated at 4^oC with a cocktail containing 1:1000 dilution of NDV polyclonal chicken serum, Nipah F and Nipah G antibodies for 1 h for detecting NiV-ndBNP. The membranes were washed again 3 times with PBST for 30 min. The membranes were allowed to react with 2 mL of chemiluminescence TMB substrate (KPL Inc.). The reaction was stopped with water before visualizing the protein bands under ChemDoc XRS (Thermo Scientific Inc.).

4.3.8 Sequence Comparison: The CT and TM domain amino acid sequences of NDV HN-NiV G and NDV F-NiV F were aligned in MegAlign (DNASTAR). NDV HN (GenBank accession #. P32884), NDV F (GenBank Accession #. P06156), NiV F (GenBank Accession #. Q9IH63) and the NiV G (GenBank Accession #. Q9IH62) were used for sequence comparisons of the CT and TM.

4.4. Results

4.4.1. Fusogenicity of NiV-ndBNP: 293T cells were transfected with pCAGGS-M (NDV), pCAGGS-F NiV and pCAGGS-G NiV plasmids and was observed for fusion every 24 h. Syncytia were evident in the cells 48 h post-transfection (Fig. 2A). Similarly, DF-1 cells transfected with NDV pCAGGS-HN, -M and -F plasmids induced syncytia appearing as multinucleated cells (Fig. 2B). It appeared that NiV-ndBNPs were more fusogenic than the NDV BNPs, which should be confirmed by future quantitative fusion studies.

4.4.2. Morphology and structure of NiV-ndBNP: 293T cells expressing M protein of NDV and F, G proteins of NiV were able to produce NiV-ndBNP with morphological characteristics similar to that of the authentic infectious virus, which was confirmed by TEM (Fig. 3A). The secreted NiV-ndBNPs were pleomorphic but generally displayed either a spherical or oval morphology (Fig. 3B to C). The spike projections on the surface of NDV M based virions were no different than authentic NiV virions (Fig. 3D). To further verify the presence of NiV F and G glycoproteins on the surface of NiV-ndBNPs, the particles were labeled with individual specific antibodies and counterstained with 12nm gold spheres conjugated to secondary antibodies (Fig. 3E).

4.4.3. Composition of NiV-ndBNPs: To determine if the purified NiV-ndBNP's were composed entirely of NDV-M and NiV-G, and NiV-F proteins, the proteins were subjected to SDS-PAGE and confirmed by immunoblot (Fig. 4, lane 2) which detected G at ~70K Da., M at ~35K Da., and F at ~40K Da along with NiV-BNP (Fig. 4, lane 1).

4.4.4. Poor sequence homology of NDV and NiV glycoproteins' CT and TM: We compared the amino acid sequences of the CT and TM of NDV (Beaudette C strain) glycoproteins (F and HN) to that of NiV (Bangladesh strain) glycoproteins (F and G) and found that only two residues in the CT (Fig. 5A to B) and seven residues in the TM (Fig. 5C to D) were similar in the F protein while six residues in CT (Fig. 6A to B) and one residue in TM (Fig. 6C to D) were similar in the attachment proteins.

4.5. Discussion

Extensive advancements have been made in the past few years towards understanding both unique and shared processes between paramyxoviruses during virus particle formation. The functions of paramyxovirus M proteins, glycoproteins, nucleocapsid proteins, and accessory proteins during virus assembly have been elucidated. We were successful in developing a proof of concept for the assembly and structural properties of NDV-based NiV BNPs (NiV-ndBNP) using the NDV M protein and NiV F and G glycoproteins. Electron microscopy revealed that the NiV-ndBNP were morphologically similar to authentic NiV virions and by immunogold labeling, we were able to demonstrate that NiV glycoproteins were incorporated into the NDV M based particles.

Paramyxovirus glycoproteins display conserved amino acid motifs in their CT and TM of glycoproteins. For example, human and bovine parainfluenzavirus 3 (hPIV3 and bPIV3, respectively) share a 5-residue sequence (PYVLT) in their F cytoplasmic tails and an 8-residue sequence (MEYWKHTN) in their HN cytoplasmic domains. Further comparison of these motifs revealed that 2 amino acids are conserved between the HNs of Sendai, hPIV1, hPIV3, and bPIV3, as are 2 amino acids (YXL) in the F protein (Takimoto et al., 1998). In addition, HN and F proteins derived from hPIV1 were incorporated into hPIV3 virions (Tao et al., 1998). Our results demonstrate that NDV M can essentially incorporate henipavirus and possibly other paramyxovirus envelope glycoproteins, despite poor sequence homology in the CT and TM domains. The mechanism(s) by which NDV M protein incorporates NiV glycoproteins need to be further explored.

Further investigations into the biological properties of the NiV-ndBNP are required to provide a clearer understanding of the events that are essential for viral particle formation and

may contribute to the development of effective antiviral strategies designed to intervene the late steps of paramyxovirus life cycles.

This report focused on the development and characterization of a flexible NDV M protein based platform to display the surface antigens of an unrelated paramyxovirus. We, therefore propose that this may serve as a promising platform for cancer therapy and vaccine/diagnostics development because 1) we have demonstrated by a simple exchange of surface antigens that our technology is flexible and can be developed to display a plethora of viral or tumor antigens on the envelope including other paramyxoviruses for cell-type specific targeting for tumor therapy or vaccine/diagnostic purposes, 2) this approach will dispense with the biosafety level-4 restrictions and bottlenecks associated with the use of live NiV.

4.6. Figures

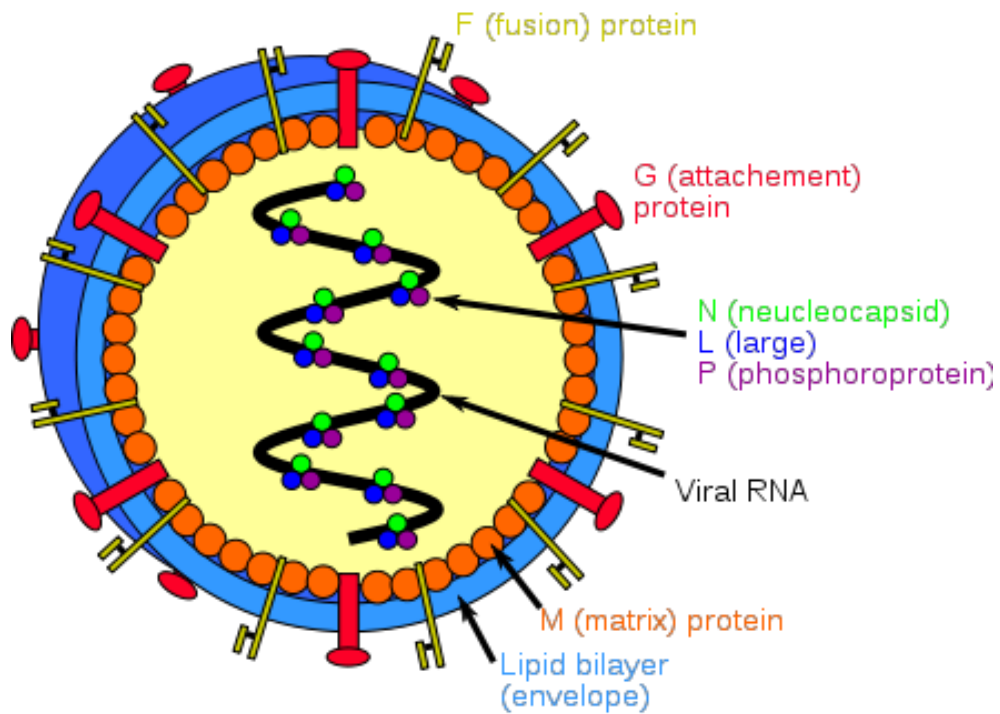
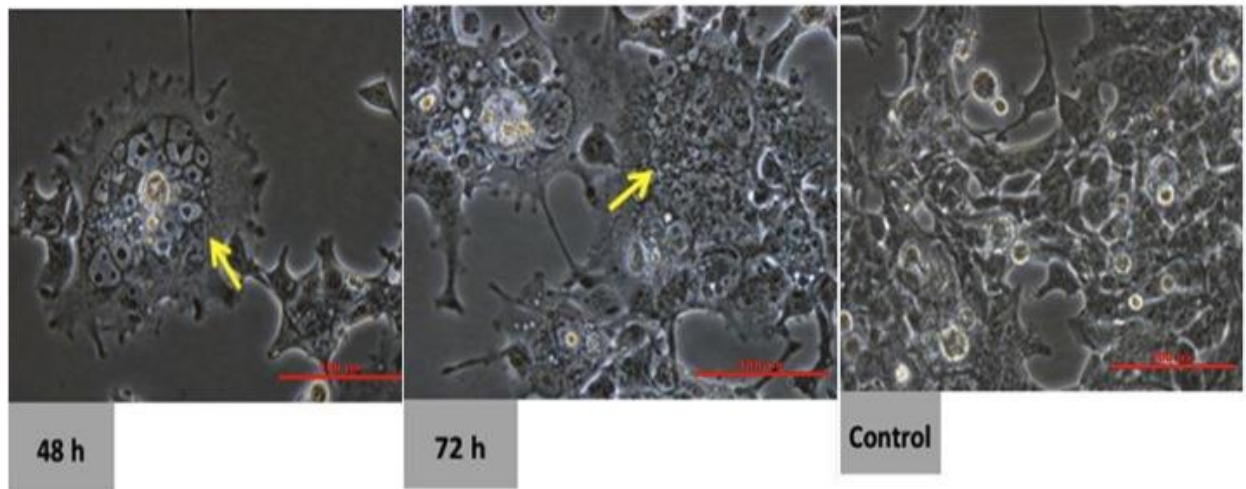


FIGURE 1. Diagrammatic representation of a henipavirus: The surface glycoproteins (G, F), Matrix (M) protein and RNP complex consisting of Nucleocapsid protein (N), Large protein (L) and Phosphoprotein (P) is shown (adapted from <http://en.wikipedia.org/wiki/Henipavirus>).

A.



B.

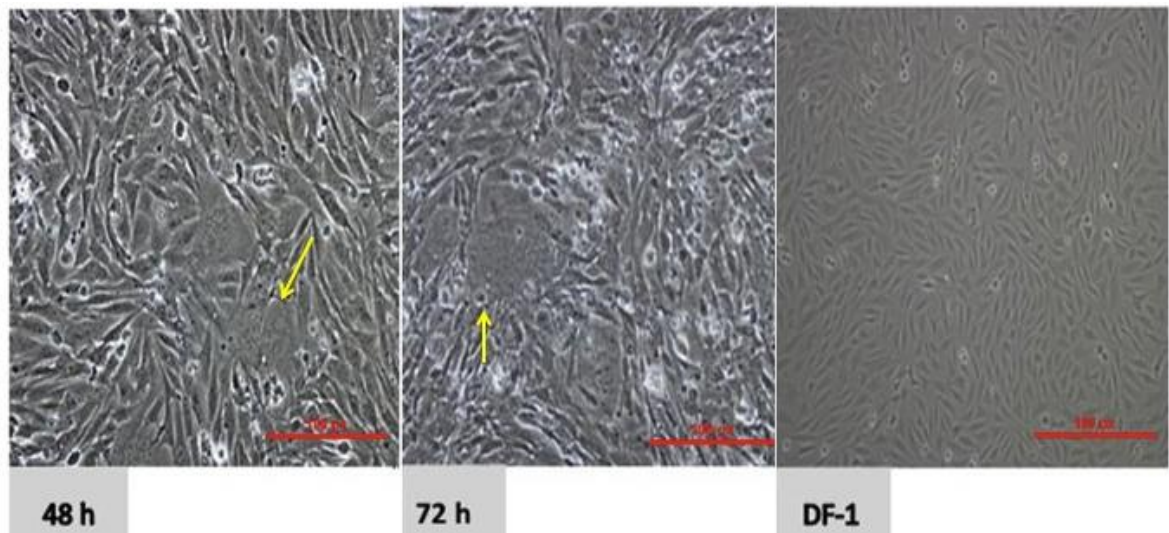


FIGURE 2. Fusogenicity of NiV-ndBNP in 293T cells: Human 293T embryonic kidney cells were transfected with pCAGGS- M (NDV), -G (NiV) and -F (NiV) plasmids. Syncytia were observed at 48 h and 72 h post-transfection by phase contrast microscopy, under 40 x magnification (A). Transfected DF-1 cells with pCAGGS-HN, -M and -F plasmids (NDV) shows syncytia at 48 h and 72 h (B). Note NiV-ndBNPs were highly fusogenic than NDV-BNPs. Arrowheads point to the syncytia.

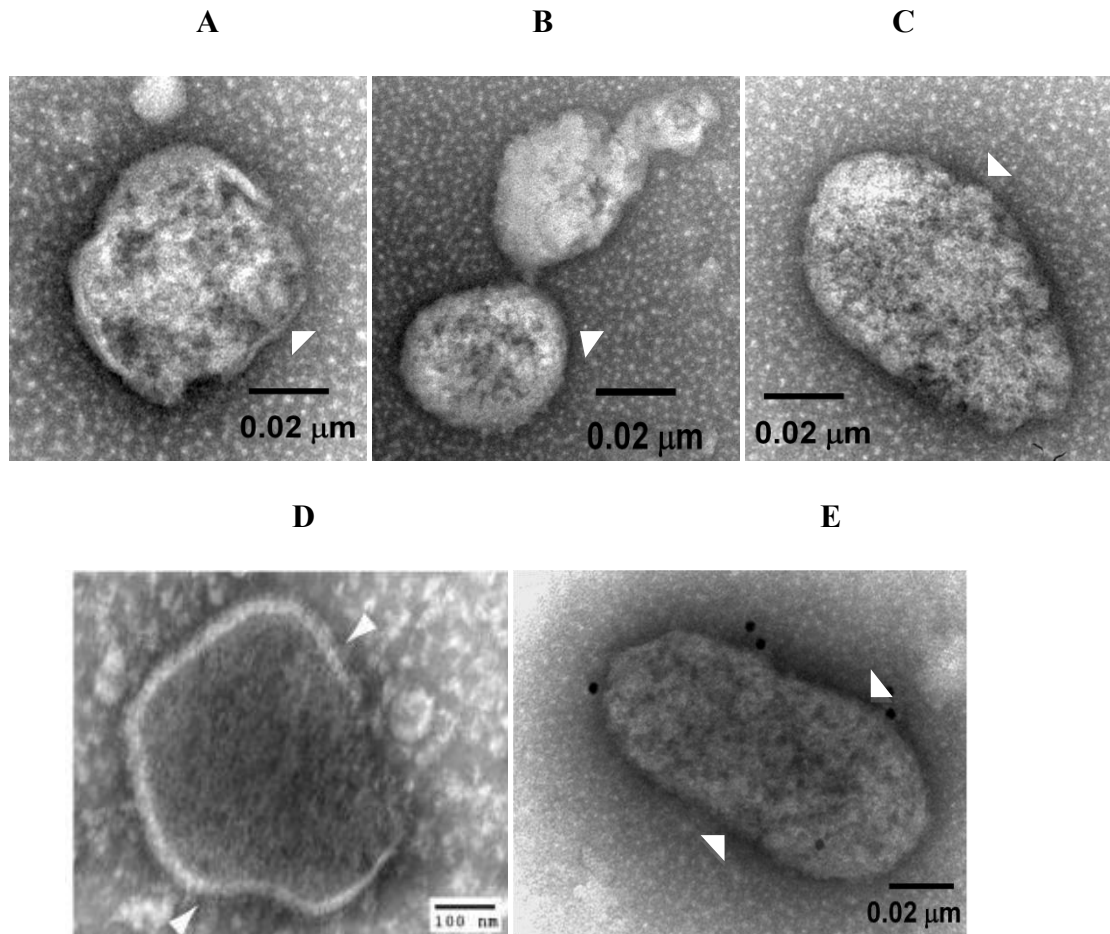


FIGURE 3. Transmission Electron Microscopy (TEM) of NiV-ndBNPs: The morphology of NiV-ndBNP (A, B, C), and the authentic NiV (Ciancanelli and Basler, 2006) virions (D) by TEM. Arrowheads indicate the NiV glycoproteins, G and/or F at the surface of the VBNPs. Immunogold labeled NiV-ndBNP (E) detected by rabbit anti-F (NiV) and anti-G (NiV) bound to 12 nm colloidal gold donkey anti-rabbit IgY antibodies.

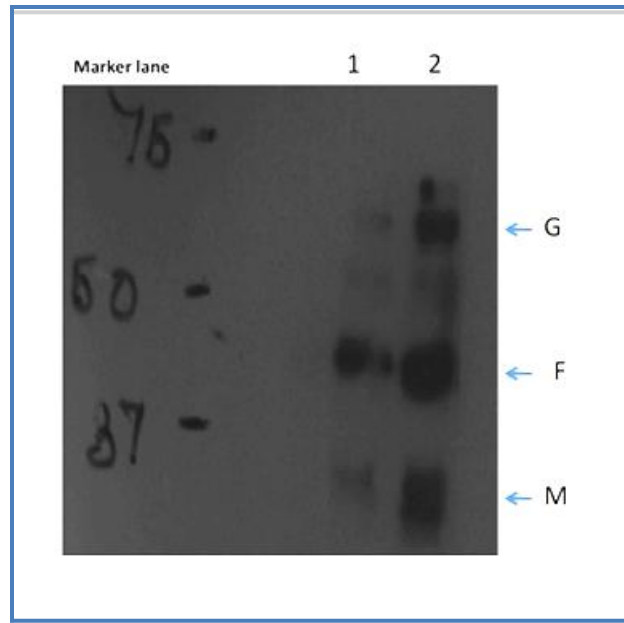


FIGURE 4. Immunoblot of NiV-BNP and NiV-ndBNP: NiV-BNP (Lane 1) and NiV-ndBNP (Lane 2) proteins were detected by SDS-PAGE and confirmed by Immunoblot using anti-NDV chicken serum and anti- NiV F, G, and M rabbit antibody which detected G at ~70K Da., M at ~35K Da., and F at ~40K Da.

A. NDV-F-CT 5..	ACYMYKQKAQKTLWLGNNNTLDQMRATTKM	553
B. NiV-F-CT 5..	EKKRNT.S-RLEDRRVRPTSSGD.YYIGT	546
C. NDV-F-TM 5..	LITYIVLTIISLVFGILSLVL	521
D. NiV-F-TM 5..	-. -. -.S.A..C I.LITFISFIIV	518

FIGURE 5. Poor sequence homology between NDV and NiV glycoproteins' CT and

TM: The amino acid sequences of the CT and TM of NDV (Beaudette C strain) glycoprotein, F were compared to that of NiV (Bangladesh strain) glycoprotein, F and only two residues in the CT (A to B) and seven residues in the TM (C to D) were homologous in the F protein. These results indicated that although the CT and TM of NDV and NiV glycoproteins had a high variability in the sequences, yet NDV M was able to incorporate F protein of NiV successfully. Homologous amino acids are highlighted in red, the numbers to the far right corner of each row indicate the amino acid sequence number and dots indicate the amino acid homology.

A. NDV-HN-CT 5..	M DRAVSQ V A E N D ERE-A K N T W R L I - - - FR	26
B. NiV-G-CT 5..	. PAENKK. RF. . TTSDKG. IPSKV. KSYYG	49
C. NDV-HN-TM 5..	IAILLT V VTLATS V ASLVYSM	48
D. NiV-G-TM 5..	FNTVIAL L GSIV I I.MNIM I I	70

FIGURE 6. Poor sequence homology between CT and TM of NDV HN and NiV

glycoprotein, G: The amino acid sequences of the CT and TM of NDV (Beaudette C strain) glycoprotein, HN were compared to that of NiV (Bangladesh strain) glycoprotein, G and only six residues in CT (A to B) and one residue in TM (C to D) were homologous in the attachment proteins. These results indicated that the CT and TM of NDV and NiV glycoproteins had a high variability in the sequences; yet, NDV M was able to incorporate G protein of NiV successfully. Homologous amino acids are highlighted in red, the numbers to the far right corner of each row indicate the amino acid sequence number and dots indicate the amino acid homology.

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4.8. Acknowledgments

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Chapter 5: General Conclusions and Future Research

The following general conclusions could be drawn from this study:

- ❖ In this study, we have established a platform by creating different NDV based nanoparticles, VBNPs, through directed expression of only three structural proteins (M, F and HN) of NDV.
- ❖ The flexibility of this platform to incorporate therapeutic transgenes was demonstrated by using a model proapoptotic protein and for ligand-directed targeting using folate conjugation on the surface.
- ❖ The VBNPs were reminiscent of authentic NDV virions in terms of size, particle morphology, and the fine structure of surface glycoprotein spikes.
- ❖ The VBNPs incorporated cellular proteins in the core.
- ❖ The VBNPs retained the biological properties of NDV virions such as infectivity, cytoplasmic distribution and fusogenicity.
- ❖ Fusion of NDV F protein transmembrane and cytoplasmic tails to foreign gene sequences will incorporate these fusion genes on the envelope of NDV.
- ❖ The VBNPs of NDV could be surface modified by conjugating them with folate for folate receptor (FR) mediated targeting.
- ❖ The proof of concept for cytotoxicity of VBNP and VBNP-VP3 in different tumor cell lines.
- ❖ Surface conjugation of folate to VBNPs targeted them specifically FR positive tumor cell lines.
- ❖ VBNPs induced killed tumor cells by apoptosis.
- ❖ VBNPs elicited proinflammatory cytokines and chemokines in tumor cells indicating they may serve as effective cancer immunotherapeutics by careful and rational design.

- ❖ Although the VBNPs were not curative in nude mouse models of human tumors, the tumor volumes over time indicated a controlled growth of tumor mass as long as the VBNPs were being administered.
- ❖ The antitumor ability of the VBNPs has to be further investigated in immunocompetent and nude mouse tumor models for dose optimization (the frequency and quantity of VBNP).
- ❖ The ability to surface modify the VBNPs by incorporating foreign genes/proteins using only the structural core M protein and CT and TM of HN protein offers for a flexible platform to carry multiple or long transgenes that would not have been possible in recombinant NDV. Our results present a novel and flexible platform for developing NDV VBNP as a powerful vaccine vector or cancer therapy agent.
- ❖ For the first time, the ability of a paramyxoviral matrix (M) protein to incorporate envelope glycoproteins from unrelated paramyxoviruses is demonstrated.
- ❖ The M protein of NDV was shown to assemble the unrelated Biosafety level-4 nipah virus (NiV) envelope glycoproteins in an unmodified form.
- ❖ NiV-ndBNP was morphologically similar to authentic NiV virions and by immunogold labeling demonstrated that NiV glycoproteins were incorporated into the NDV M based particles.
- ❖ This novel approach will dispense with the biosafety level-4 restrictions and bottlenecks associated with the use of live NiV.

Future directions:

- ◆ This platform VBNP technology is very promising and flexible to engineer various therapeutic transgenes and receptor specific targeting.
- ◆ It would be interesting to study the ability of these VBNPs to carry fusion products of tumor -associated antigens to elicit effective antitumor immunity.
- ◆ The ability to engineer the fusion protein of NDV for protease specific targeting offers an attractive target for cell type specific delivery of VBNPs.
- ◆ Dose optimization studies in nude mouse models of xenotransplanted tumors.
- ◆ The ability of VBNPs to elicit NK-cell mediated antitumor immunity could be studied in nude mouse models of xenotransplanted human tumors.
- ◆ The efficacy of VBNPs in immunocompetent preclinical tumor models
- ◆ The nature and composition of cellular proteins in the VBNPs need to be identified which would provide insights into the assembly and sorting of cellular proteins.
- ◆ The VBNP platform provides an opportunity to understand the assembly and budding of paramyxoviruses and afford development of intervention strategies to intervene in the late stages of paramyxovirus life cycle.
- ◆ The mechanism by which NiV glycoproteins is able to get incorporated into NDV M based particles needs to be further explored.
- ◆ Further research is needed to study the biological properties of the NiV-ndBNP and to understand their properties to provide a clearer understanding of the events that are essential for viral particle formation and may contribute to the development of effective antiviral strategies for this BSL-4 agent.
- ◆ Future research may be directed to receptor-mediated targeting to ephrin B receptors and oncolytic efficacy of NiV-ndBNP and NiV BNPs using *in vitro* and *in vivo* tumor models.