PREVENTION OF RESPIRATORY SYNCYTIAL VIRUS INFECTION VIA METHOXYPOLY(ETHYLENE GLYCOL)-MODIFICATION OF THE VIRUS OR ITS HOST CELL

by

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ABSTRACT

Respiratory Syncytial Virus (RSV) causes significant morbidity and mortality in humans. To date, no vaccines or effective treatments exist for this serious respiratory disease. Current prophylactic therapy is limited to at-risk neonates due to its high cost, and involves the administration of anti-RSV immune globulins that are ~50% effective. To attenuate or inhibit RSV infection, we hypothesized that bioengineering of either the virus particle or host cell with methoxypoly(ethylene glycol) [mPEG] would prevent viral infection. Our specific objectives were to evaluate the effects of grafting concentration, linker chemistry, polymer length, and cell polarization on viral infection and propagation.

Modification of either the virus or host cells with mPEG prevented RSV infection in a dose- and size-dependent manner. For virus modification, short chain polymers (2 kDa) were significantly more effective than long chain polymers (20 kDa). For example, plaque assays demonstrated that RSV modification with 5 mM, 2 or 20 kDa mPEG resulted in a 100 and ~82% plaque reduction, respectively. In contrast, when small polymers were used to modify the host cell they provided no protection, while long chain polymers effectively prevented infection. For example, at 48 hours post-infection at a multiplicity of infection of 0.5 and grafting concentrations of 5, 7.5, and 15 mM, 20 kDa mPEG decreased infection by 45, 83, and 91%, respectively. However, these grafting concentrations of the 2 kDa mPEG resulted in ~0% reduction. Importantly, with both viral and host cell PEGylation strategies, moderate to high grafting concentrations of the appropriate polymer species were able to provide near complete protection against infection in both non-polarized and polarized cells.

In conclusion, mPEG-modification of RSV or the host cell are highly effective methods for preventing viral infection. Our findings indicate that the length of grafted polymer must be matched to the size of particle targeted for modification. Consequent to the high efficacy of both

PEGylation approaches, future studies should evaluate mPEG-modified RSV as a vaccine strategy, and mPEG-grafting to the nasal epithelium as a prophylactic therapy.

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ANOVA	Analysis of variance
APC	Antigen-presenting cells
ATCC	American Type Culture Collection
BTCmPEG	Benzotriazole carbonate-mPEG
CR1	Complement receptor 1
cm	Centimeter
CmPEG	Cyanuric chloride mPEG
CMV	Cytomegalovirus
Da	Dalton
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
F protein	Fusion protein
FBS	Fetal bovine serum
FDA	Food and Drug Administration
g	Gram
G protein	Attachment protein
GAGS	Glycosaminoglycans
GFP	Green fluorescent protein
GFP-RSV	Green fluorescent protein expressing-RSV
GVHD	Graft versus host disease
HBD	Heparin binding domains

HIV	Human immunodeficiency virus
HS	Heparan sulfate
IL	Interleukin
IFN	Interferon
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
L	Liter
LRTI	Lower respiratory tract infection
M protein	Matrix protein
MAV	Mouse adenovirus
MCP-1	Monocyte chemotactic protein-1
MDCK	Madin-Darby canine kidney cells
MEM	Minimal essential media
mg	Milligram
MIP-1 α	Macrophage inflammatory protein-1 alpha
mL	Milliliter
mm	Millimeter
mM	Millimolar
MOI	Multiplicity of Infection
mPEG	Methoxypoly(ethylene glycol)
mRNA	Messenger ribonucleic acid
N protein	Major nucleocapsid protein
NK cells	Natural killer cells
nm	Nanometer

NS1	Nonstructural protein 1
NS2	Nonstructural protein 2
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PEG-ADA	Pegylated adenosine deaminase
pfu	Plaque forming units
PTA	Phosphotungstic acid
PVM	Mouse pneumovirus
R_g	Radius of gyration
R_g max	Maximum radius of gyration
RANTES	Regulated on Activation, Normal T Expressed and Secreted
RBC	Red blood cells
RCV	Rat coronavirus
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RSV-IGIV	RSV Immune globulin intravenous
SCmPEG	Succinimidyl carbonate mPEG
SE	Standard error
SeV	Sendai virus
SH	Small hydrophobic protein
SPAmPEG	N-hydroxysuccinimidyl ester of mPEG proprionic acid
SPSS	Statistical products and services solutions software
SV40	Simian virus 40
SVAmPEG	Succinimidyl valerate mPEG
$TCID_{50}$	Tissue culture infection dose 50%

TEM	Transmission electron microscopy
Th	T helper cell
TLR-4	Toll-like receptor 4
TmPEG	Tresyl-mPEG
TMEV	Theiler's murine encephalomyelitis virus
TNF	Tumor necrosis factor-alpha
μg	Microgram
μL	Microliter
μm	Micrometer
URTI	Upper respiratory tract infection
VP1	Viral protein 1
WBC	White blood cells
WHO	World Health Organization

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CHAPTER 1: INTRODUCTION

1.0 OVERVIEW

Respiratory syncytial virus (RSV) is a highly contagious respiratory virus that causes significant morbidity and mortality in humans. Currently, there are no treatments, vaccines, or cost-effective prophylactic therapies capable of diminishing the disease burden of this virus. Consequently, new approaches are needed to prevent RSV infection and disease.

Methoxypoly(ethylene glycol) [mPEG] is a non-toxic, FDA approved polymer that can be grafted to proteins on biological surfaces. Previous studies on mPEG-modification of simian virus 40 (SV40) have shown that mPEG grafting directly to the virion inhibits viral infection. Additionally, mPEG-modification of host cell surfaces was also shown to inhibit viral invasion of several viruses including SV40, Theiler's murine encephalomyelitis virus (TMEV), mouse adenovirus (MAV), and rat coronavirus (RCV). Thus, we sought to evaluate mPEG-modification in the context of RSV and explore the relationship between polymer linker chemistry, grafted polymer density and polymer length, on the extent of virus inactivation and host cell protection.

1.1 RESPIRATORY SYNCYTIAL VIRUS (RSV)

1.1.1 Historical Background

RSV was first isolated in 1956 from a colony of chimpanzees suffering from cold symptoms, and was originally named "chimpanzee coryza agent" (1). One year later, a similar virus was isolated by Robert Chanock from infants suffering with respiratory illness. Based on syncytia formation in tissue culture and similarities between the monkey and human isolates, the virus was named "respiratory syncytial virus (2). Subsequently, through several epidemiological

studies, RSV quickly became recognized as the most prominent cause of bronchiolitis and a major cause of pneumonia in the pediatric population (3, 4). More recently, RSV has been recognized as a cause of the common cold in adults (5-8) and as a serious cause of morbidity and mortality in the elderly and the immunocompromised (9-19). At present, despite nearly 40 years of research, no effective treatments or cost-effective prophylactic therapies have been developed and there is a continued demand for novel prophylactic therapies against RSV.

1.1.2 RSV Disease and Epidemiology:

RSV causes significant disease in infants, children, adults, the elderly, and the immunocompromised, and RSV infection is the leading cause of infant hospitalization in the developed world (3, 9, 10, 17, 18, 20). Hence, the World Health Organization (WHO) recognizes RSV as one of the most important pathogens for vaccine or prophylactic therapy development (4).

RSV infects nearly all children within the first two years of life with infection rates of 66% and 80% during the first and second years, respectively (8). In most children, RSV infection is limited to the upper respiratory tract (see Figure 1.1) and results in a "common cold". Signs and symptoms include clear rhinorrhea, sneezing, fever, productive cough, and diminished appetite (21, 22). In some children, RSV spreads to the lower respiratory tract causing severe infection and the clinical manifestations of bronchiolitis and/or pneumonia. Bronchiolitis is characterized by wheezing, hyperinflation, and atelectasis, while pneumonia is defined by alveolar consolidation and exudation (20).

Approximately 1-3% of infants develop severe lower respiratory tract infection requiring hospitalization (23). Consequently, in the United States, 60,000–100,000 infants are hospitalized each year at an estimated annual cost of $341-449 million (23, 24). Severe RSV infection has a mortality rate of 8.3–10.4 deaths per 100,000 infants, with male infants 1.3-1.4 times more likely

to develop severe disease than female infants (25). Other risk factors for severe infection include premature birth, bronchopulmonary dysplasia, congenital heart disease, chronic respiratory disease (*e.g.* cystic fibrosis), and immunodeficiency (*e.g.* chemotherapy or HIV) (26-28).

Following resolution of the initial infection, hospitalized children are predisposed to the development of recurrent wheeze and asthma-like symptoms (29-31). Furthermore, children (or adults) do not develop protective or durable immunity against RSV; therefore, repeat infections occur in childhood and throughout life. In older children and adults, repeat infection is predominantly limited to the upper respiratory tract and again manifests as a common cold (5-7). Thus, RSV infection in these populations seldom requires hospitalization and is largely unrecognized and undiagnosed (7, 32).

In contrast, in the elderly and immunocompromised adults, RSV is again able to spread to the lower respiratory tract and cause pneumonia, (9-11). As a result, in the elderly, RSV is estimated to have similar morbidity and mortality rates to influenza A with an estimated 8% mortality rate in hospitalized RSV infected patients (10). In immunocompromised patients, RSV has an estimated mortality rate of 20-100%, with a greater than 80% mortality rate once pneumonia develops (13-19, 33, 34). In these patients, RSV is particularly difficult to control as patients have a dramatically reduced ability to mount an immune response and anti-RSV treatments are limited and relatively ineffective. Consequently, RSV infection is largely uncontrolled and pneumonia can be the direct cause of death or can be a confounding factor in combination with the underlying disease (11).

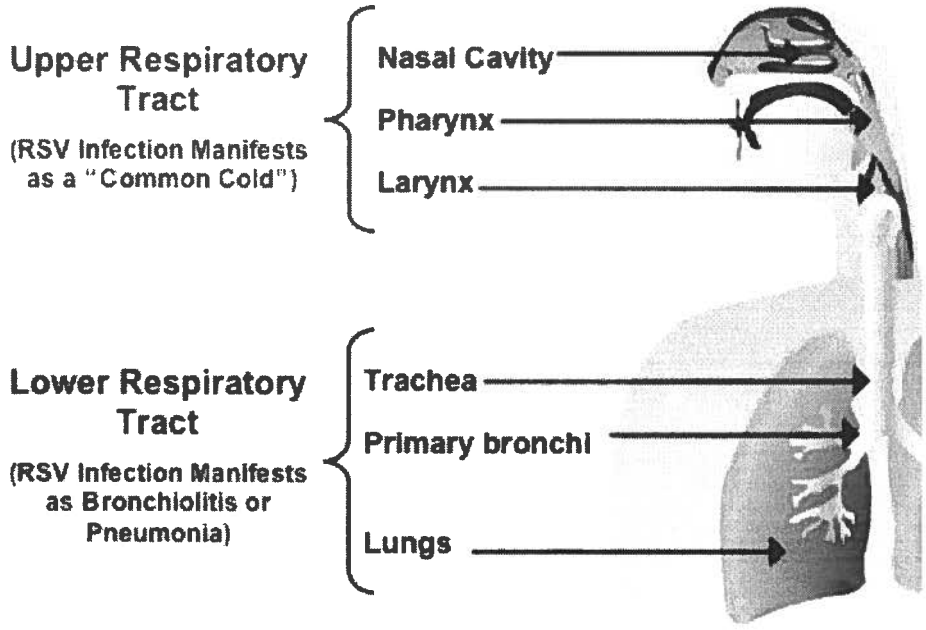


Figure 1.1. Schematic of the Upper and Lower Respiratory Tract. RSV infection of the upper respiratory tract results in a "common cold", while infection of the lower respiratory tract results in more severe disease. Lower respiratory tract infection manifests as bronchiolitis or pneumonia. Modeled from (21).

1.1.3 Treatment of Severe RSV Infection

For infants and hospitalized patients with severe RSV infection, the mainstay of therapy is supportive treatment. Patients are kept warm, given intravenous fluids, and their arterial blood gas levels are monitored. Infants showing signs of respiratory failure are intubated and mechanically ventilated (35). Several pharmacological therapies exist to treat severe RSV infection, however they are of little or questionable benefit (36). These therapies include bronchodilating agents, epinephrine, corticosteroids, and the anti-viral drug Ribavirin. Bronchodilating agents are delivered as an aerosol and are used to treat wheezing. Compounds that have been evaluated include albuterol, metaproterenol, sabutamol, and ipratropium. Given that many of the studies evaluating these agents involved small numbers of patients, meta-analyses of 24 published studies has been performed. This analysis found that 50% of the studies showed a positive effect while the remaining 50% found no effect (37). Furthermore, the positive studies were misleading as only 30-50% of the patients in these studies showed a response to the bronchodilators. However, despite this weak evidence, bronchodilators are currently recommended on a trial basis, with therapy being continued only if the patient responds to the initial dose (37).

Epinephrine has also been used as a treatment for severe RSV infection. Epinephrine is administered either as an aerosol or by injection, and is given to counteract interstitial edema. Eight of 10 small single center studies reviewed by Black (37) showed some positive effect of epinephrine, based on decreased respiratory distress and wheezing (38). However, two more recent multi-center randomized double-blind control trials found that epinephrine treatment did not significantly decrease the length of hospital stay (39). Thus, it appears that epinephrine may provide some relief of clinical symptoms, but it does not dramatically alter disease progression.

Due to the inflammatory nature of severe RSV infection, inhaled and systemic corticosteroids have also been evaluated as treatments. Unfortunately, large-scale studies have

shown that the rate of clinical recovery, respiratory distress scores, and requirements for supportive therapy were not significantly different between infants treated with corticosteroids and placebo treated infants (40-42). Thus, corticosteroids are considered to be of little or no benefit to patients with severe RSV infection.

Currently, Ribavirin is the only anti-viral agent licensed by the US Food and Drug Administration (FDA) against RSV. Ribavirin is a synthetic guanosine nucleoside analogue and is administered as an aerosol for 12-20 hours/day (43). Early clinical trials were promising and showed positive results; however, larger subsequent studies showed that ribavirin did not reduce mortality or duration of hospitalization in infants with bronchiolitis (44). Consequent to these findings, ribavirin is now administered only to infants with exceptionally severe bronchiolitis or with substantial comorbid conditions (45).

In summary, several pharmacological therapies have been evaluated against RSV. While some therapies may alleviate symptoms, no therapy is capable of altering the progression of disease or dramatically improving patient outcome. Consequently, anti-RSV prophylaxis (discussed below) in high-risk neonates remains the clinical standard and the development of effective anti-RSV prophylactic strategies remains the most suitable means of limiting RSV-associated morbidity and mortality.

1.1.4 Prophylaxis of RSV Infection

1.1.4.1 RSV Vaccines

In the 1960's, following the success of the killed polio virus vaccine, clinical trials using a formalin-inactivated RSV vaccine were conducted with children aged 2 months to 9 years. Unfortunately, upon subsequent natural RSV infection, 80% of the vaccinees were hospitalized compared to 5% of the controls, and two vaccinated infants died (46). As a result, RSV vaccine development has been greatly hindered due to safety concerns.

However, vaccine research has continued and several strategies have been evaluated. These strategies include live-attenuated RSV vaccines, protein subunit vaccines, DNA vaccines, and reverse engineered RSV vaccines. For live-attenuated RSV preparations, the virus strains have either been over or under attenuated producing a weak immune response or causing overt disease (47-49). More recently, protein subunit vaccines directed against the RSV F-protein have also been evaluated. While these preparations have been shown to be safe they did not induce robust protective immunity (50-54). DNA vaccines have been plagued by similar problems. These preparations have also been shown to be weakly immunogenic and thus require multiple boosters to elicit an immune response (55).

Currently, the most promising vaccine strategy is the development of reverse engineered RSV vaccines. Using reverse genetics, virus strains can be generated such that specific viral genes are missing. To date, several strains have been developed and shown to be safe; however, the level of protection conferred has yet to be demonstrated (55). With this uncertainty and given that traditional vaccine approaches have already been extensively explored, there is a continued demand for novel approaches to RSV inactivation for subsequent vaccine development.

1.1.4.2 Passive Immunization: Prophylaxis with Anti-RSV Antibody Preparations

As a result of the challenges of developing a RSV vaccine, prophylactic research has focused on the development of anti-RSV immunoglobulin products. Two products are currently licensed for prophylactic treatment of RSV infection: 1) RSV immune globulin intravenous (RSV-IGIV)(RespiGam) and 2) Palivizumab (Synagis). RSV-IGIV was introduced in the 1990's and is a polyclonal antibody preparation produced from the sera of adult humans (56). Palivizumab is a humanized mouse monoclonal antibody directed against the RSV F-protein and was approved by the FDA in 1998. For prophylactic treatment, one of these preparations is

given once a month for 5 months during the RSV season (December to April). RSV-IGIV is given intravenously while Palivizumab is administered as an intramuscular injection. Clinical trials with RSV-IGIV and Palivizumab have shown a reduction in hospitalization rates by 41% and 51%, and the total number of days spent in the hospital by 53% and 58%, respectively (56, 57). Given that both immunoglobulins have similar efficacy, the American Academy of Pediatrics recommends that Palivizumab be used instead of RSV-IGIV due to the ease of intramuscular injection and decreased risk of transfer of blood borne pathogens (58). The Academy further recommends that prophylaxis be limited to children at high risk (having two or more risk factors) for severe infection as prophylactic treatment is extremely expensive (59). For example, in Canada treatment cost is between $7-9000 per patient and during the 2006-2007 RSV season Palivizumab prophylaxis cost a total of $32.5 million (60). Consequently, the vast majority of infants do not receive prophylactic treatment and new cost-effective therapies are needed.

1.1.5 RSV Biology and Virion Structure

1.1.5.1 Spread, Transmission, and Infectious Dose of RSV

RSV is spread by transmission or self-inoculation of large-particle aerosols on to the nasal mucosa or conjunctivae. RSV does not spread by aerosolization of droplet nuclei, thus sitting in the same room with an infected individual does not result in transmission of the virus (61). RSV infection occurs when an individual contacts contaminated secretions on clothing or toys, and then touches their nose, eyes, or face. RSV is particularly contagious as it can survive for several hours on inanimate objects such as linens, tissue, and countertops (61). Thus frequent hand washing and education remain the most effective methods of prophylaxis against RSV (62).

The infectious dose of RSV in infants is unknown and infectious dose titration studies have not been performed in humans. Shortly after the discovery of RSV, however, studies were

conducted to determine if RSV could induce disease in healthy adults. In these studies, a dose of RSV Long Strain A with an endpoint dilution titer between 160-640 tissue culture infection dose 50% ($TCID_{50}$) was given to 41 men aged 21-35 years. $TCID_{50}$ is defined as the inverse dilution of a RSV per mL of solution that produces cytopathic effects in 2 of 4 (50%) cultures and can be converted to plaque forming units (pfu) by multiplying by 0.7 (63). Thus, this virus dose corresponded to approximately 112 - 448 pfu (63) and was shown to produce clinical disease characterized by nasal discharge and malaise in 20 of the 41 volunteers (64). Given that all subjects were in good health and had pre-existing anti-RSV antibodies, it is likely that the infectious dose in infants, the elderly, and immunocompromised patients, is lower than suggested by this study.

1.1.5.2 Classification of RSV

RSV circulates in the human population as one serotype with two major strains, A and B (4). These two strains differ predominantly in the sequence and structure of the G-protein (see section 1.1.5.3). Both strains have been associated with severe disease, however, the A strain appears to cause severe infection in a higher proportion of infants (65). In the laboratory, two strains are used almost exclusively and include strain A2 and Long Strain A.

RSV belongs to the family *Paramyxoviridae* of the order *Mononegavirales* (4). The *Paramyxoviridae* is divided into two subfamilies: *Pneumovirinae* and *Paramyxovirinae*. The *Paramyxovirinae* subfamily includes viruses such as Sendai virus, measles, mumps, and human parainfluenza virus, while the *Pneumovirinae* is composed of the human strains of RSV (A and B), bovine RSV, metapneumovirus, and mouse pneumovirus (4). Members of the *Paramyxoviridae* share several features. All members are encapsulated in a bilipid envelope acquired by budding from the host cell surface and all members infect host cells by fusing with their plasma membrane. Viruses of the *Paramyxoviridae* also have a non-segmented, single-

stranded, negative-polarity RNA genome that is found exclusively in an RNase resistant helical nucleocapsid with the viral polymerase. The RNA genome is transcribed in a sequential stop-restart mode producing subgenomic mRNA's, and the virus replicates in and is restricted to the cytoplasm of the host cell (4).

1.1.5.3 RSV Structure

The RSV virion has an irregular spherical shape when visualized under an electron microscope, with particles ranging in size from 150-300 nm in diameter. Each virion consists of a nucleocapsid contained in a bilipid envelope and has a single functional copy of the RNA genome. The RSV genome encodes 11 proteins and is approximately 15.2 kb in length. The A2 strain genome is 15,222 nucleotides long, while the Long Strain A genome contains 15,226 nucleotides (4). The virion envelope contains three virally encoded transmembrane surface glycoproteins: 1) the attachment (G) protein, 2) the fusion (F) protein, and 3) the small hydrophobic (SH) protein (see Figure 1.2). On the virion surface, these glycoproteins form "spikes", 11-20 nm long and 6-10 nm apart (4). The G protein facilitates attachment to the host cell while the F protein is responsible for fusion with the host cell membrane and viral penetration. Deletion of the F protein abrogates infection. In contrast, deletion of the G protein does not prevent infection, but does reduce the ability of the RSV virion to bind, invade, and assemble itself within the host cell (66). Both the G and F proteins are known to interact with a number of cell surface proteins (discussed in section 1.1.5.5), but their key host membrane binding targets are unknown. The SH protein is a short integral membrane protein with unknown function, and deletion of this gene does not alter the replication efficiency and infectivity both in vitro and *in vivo* (67).

The matrix (M) protein is also encoded by the viral genome and is thought to form a layer on the inner surface of the virion envelope. During host cell infection, the M protein

accumulates at the plasma membrane where it interacts with the F protein and other factors to assemble the RSV virion. The M protein also appears to negatively regulate transcription as removal of this protein from the nucleocapsid results in enhanced transcriptional activity (4).

The RSV nucleocapsid is a symmetrical helix, 12-15 nm in diameter, and is comprised of 4 proteins. These proteins include: 1) major nucleocapsid (N) protein, 2) phosphoprotein P, 3) the antitermination factor M2-1, and 4) the viral polymerase (L) protein (4). The N protein binds tightly to genomic RNA to form the RNase resistant nucleocapsid and the phosphoprotein P is a chaperone for protein N. The L protein is the viral polymerase and the M2-1 protein is an essential transcription factor that promotes transcription of the viral genome (4). Together, the N, P, and L proteins are sufficient and necessary to direct RNA replication; however, for full transcriptional activity, and thus virus viability, the M2-1 protein is required. The RSV genome also encodes the M2-2 protein and the nonstructural proteins NS1 and NS2. The M2-2 protein helps regulate transcription, however, it is also thought to play a role in shifting the balance of RNA synthesis from transcription to RNA replication (4). The NS1 and NS2 proteins were originally of questionable function, though, *in vitro* and *in vivo* studies showed that recombinant viruses lacking these genes replicated less efficiently (68, 69). Subsequent studies evaluated the role of NS1 and NS2 in the context of the innate immune response and demonstrated that NS1 and NS2 decrease host IFN-alpha/beta expression early during RSV infection (70, 71).

1.1.5.4 Host Cell Proteins in the Viral Lipid Envelope

Upon maturation, RSV virions assemble and bud from lipid rafts in the host cell plasma membrane (72). During viral replication (see section 1.1.5.6), these lipid rafts become enriched with the F, G and SH viral surface proteins (73). However, some host cell proteins are also incorporated into the viral envelope. The known host cell proteins in the RSV envelope included caveolin-1, CD55, and CD59 (72-74). Other host cell proteins are also thought to be present in

the envelope; however, they have not yet been identified. Caveolin-1 is a protein present in lipid rafts and may facilitate RSV in binding to and fusing with lipid rafts of uninfected cells (75). CD55 and CD59 are complement regulatory proteins that may inhibit complement media lysis of virus particles (74).

Importantly, these proteins represent a relatively small component of the total envelope protein content. For example, using a combination of immunostaining and scanning electron microscopy, CD55 and CD59 were shown to account for between 5-15% of the protein content relative to levels of the RSV G protein (74). Thus, while host proteins are a feature of the viral envelope and may potentially influence both viral infection and the host immune response, the majority of proteins contained within the envelope are viral encoded surface proteins.

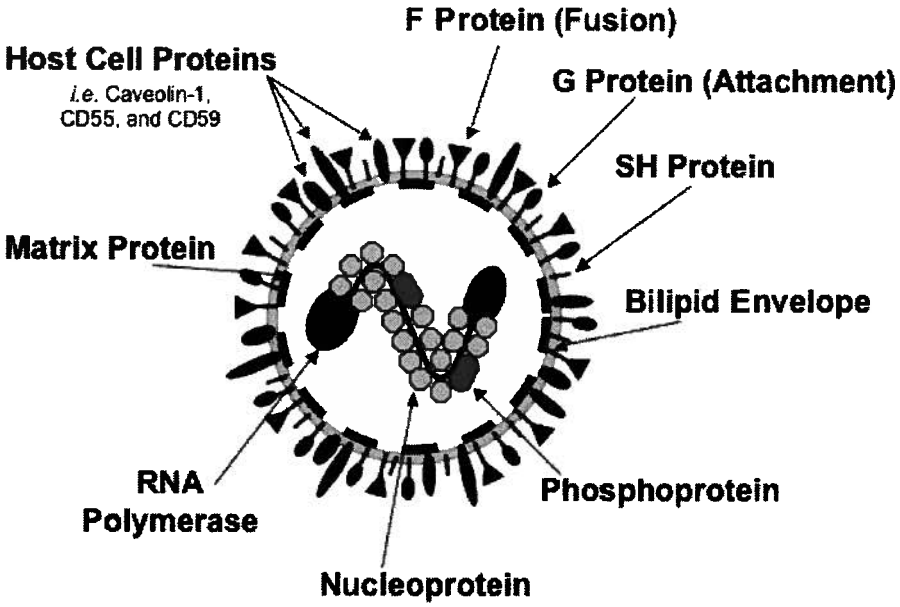


Figure 1.2. Schematic of the RSV Virion. The RSV virion is encapsulated in a bilipid layer acquired during budding from the host cell membrane. On the virion surface are the viral encoded G, F and SH proteins, as well as several host cell proteins including caveolin-1, CD55 and CD59. The viral encoded G and F proteins mediate host cell attachment and membrane fusion, respectively. The SH protein has no known function. Modeled from Hall CB, 2001 (7).

1.1.5.5 RSV Life Cycle

In humans, RSV infects polarized, ciliated, airway epithelial cells (76); however, in tissue culture, RSV readily infects several non-polarized cell lines including Hep-2, HeLa, A549, and Vero cells, and some polarized cell lines such as Madin Darby canine kidney (MDCK) and Vero 1008 cells (77). The RSV life cycle is illustrated in Figure 1.3 and begins with attachment of the virus to the host cell membrane. The G protein on the viral envelope mediates attachment and the F protein subsequently binds to an unidentified high affinity receptor and induces fusion of the viral envelope and cell membrane (note: specific cell-virus protein interactions are discussed in section 1.1.5.6). Upon fusion, the viral nucleocapsid is injected into the cytoplasm of the host cell and the M protein dissociates from the nucleocapsid rendering the genome active for transcription and replication (4). Viral replication and protein synthesis is restricted to the cytoplasm and there is no nuclear involvement.

The viral polymerase L protein sequentially transcribes all 10 genes producing positive polarity mRNA's that act as templates for translation of viral proteins (4). The polymerase proceeds down the viral genome in the 3' to 5' direction, first producing the NS1 and NS2 mRNA and then proceeding to the N, P, M, SH, G, F, M2, and L genes. Of note, the mRNA for M2 encodes both the M2-1 and M2-2 proteins. Located at the end of each gene sequence is an intergenic "pause" or stutter site. At this site, the polymerase-viral RNA interaction becomes unstable and the polymerase either dissociates from the template and restarts at the 3' end of the genome, or releases the mRNA and proceeds to transcribe the next gene. As each gene is followed by a stutter site, the viral polymerase has a higher probability of dissociating as it sequentially transcribes genes further down the genome. Thus, mRNA is synthesized in decreasing amounts in the 3' to 5' direction. Once an mRNA template is transcribed, host cell ribosomes and tRNA are then used to translate each mRNA and produce the viral proteins (78). The viral proteins, especially the G and F proteins, undergo glycosylation and post-

transcriptional folding in the golgi apparatus and are subsequently transported to the cell membrane. The G protein is synthesized in both a soluble excreted form and a membrane bound form that is incorporated into the virion (4). The secreted form is thought to bind circulating RSV antibodies, therefore allowing enhanced survival of progeny virions (4).

Eventually, high levels of the M2-2 protein are produced and induce a shift in the activity of the polymerase promoting RNA replication and synthesis of full-length viral genomes. Full length positive sense RNA templates are produced that are then transcribed to produce the negative sense viral genomic RNA. The N protein is now present at high levels binds to the full-length copies of the negative sense RNA genome and in coordination with the M protein render RNA genome transcriptionally inactivate. The N and P proteins and the viral polymerase are then packaged into a nucleocapsid with a single copy of the viral genome. The M protein subsequently coordinates the assembly of the various proteins and a nucleocapsid into a virion at lipid rafts in association with lipid rafts at the cell surface. Multiple virions are assembled within a cell and are released by budding from the plasma membrane on the apical surface of the cell (4). After budding new virions infect adjacent and neighbouring cells allowing further viral proliferation/propagation.

Following infection of HeLa cells, viral antigens can be detected in cell culture 9 hours after infection, and the release of infectious particles begins by 10-12 hours post-infection. Release of progeny virus peaks after 24 hours and continues until the host cell dies at 30-48 hours post-infection (79).

In vitro, during RSV infection non-polarized cells express F-protein on both the apical and basolateral surfaces. Consequently, the lateral surfaces of infected cells fuse with adjacent cells and form syncytia. In contrast, in RSV infected polarized cells F protein expression is limited to the apical surface. As a result, infected cells are not able to fuse with neighboring cells and syncytia formation does not occur (76, 77, 80).

1.1.5.6 RSV-Host Cell Surface Protein Interactions

At the cell surface, the G and F proteins of the RSV envelope interact with several host cell surface molecules. The most significant interaction occurs between RSV and cell surface glycosaminoglycans (GAGs), heparan sulfate (HS) and chondrotin sulfate B, with RSV showing higher affinity binding to HS (81). RSV can also bind heparin. Heparin is a soluble GAG similar in structure to HS; however, it is only produced and released by mast cells (82).

HS is a long unbranched molecule that projects 40-160 nm from the cell membrane, and therefore, is a prominent feature on the cell surface (82). The RSV G and F proteins interact with HS through heparin binding domains (HBD) contained within their protein sequence (83, 84). These binding domains interact with HS via electrostatic interactions between the negatively charged sulfate groups on HS and the positively charged amino acids within the HBDs.

The interaction between RSV and HS is considered the initial binding step during RSV infection. This binding is most likely followed by a second high affinity interaction between the G and F proteins and an unidentified cell surface receptor (81). This idea is supported by the findings that RSV infection is reduced but not abolished when GAG-deficient cells (via knock out or enzymatic treatment) are challenged with RSV (81).

The G protein has also been shown to interact with the cell surface proteins annexin II and the chemokine receptor CX3CR1, while the F protein can interact with RhoA and toll-like receptor (TLR)- 4. These interactions are thought to represent that of co-receptors that act with the unknown RSV receptor or may modulate the host immune response. For example, RSV replicates with decreased efficiency in TLR-4 deficient mice, while wild type RSV, relative to recombinant RSV lacking G protein, shows reduced trafficking of CX3CR1 containing cytotoxic T cells to the lung (85, 86). RhoA over-expression enhances RSV infection and syncytia formation *in vitro* suggesting it may have a co-receptor like function. However, while RhoA is

expressed on the cell surface and within the cytoplasm, it remains unclear whether this interaction occurs at the cell membrane or in the cytoplasm (87). Annexin II and the RSV G protein interact at the cell surface, and the finding that pre-incubation of RSV with annexin II results in a 40% reduction in the number of cells infected suggests that annexin II likely has a role as a co-receptor (88).

After viral binding to HS and, subsequently, the unknown RSV receptor, the RSV F protein undergoes a conformation change, pulling the virus and cell membranes into close proximity. The F protein then inserts into the host cell membrane and fusion occurs (4). Binding is a slow process while fusion occurs quickly (89), however, inhibition of attachment or the conformation change of the F-protein, both represent potential mechanisms to inhibit viral infection.

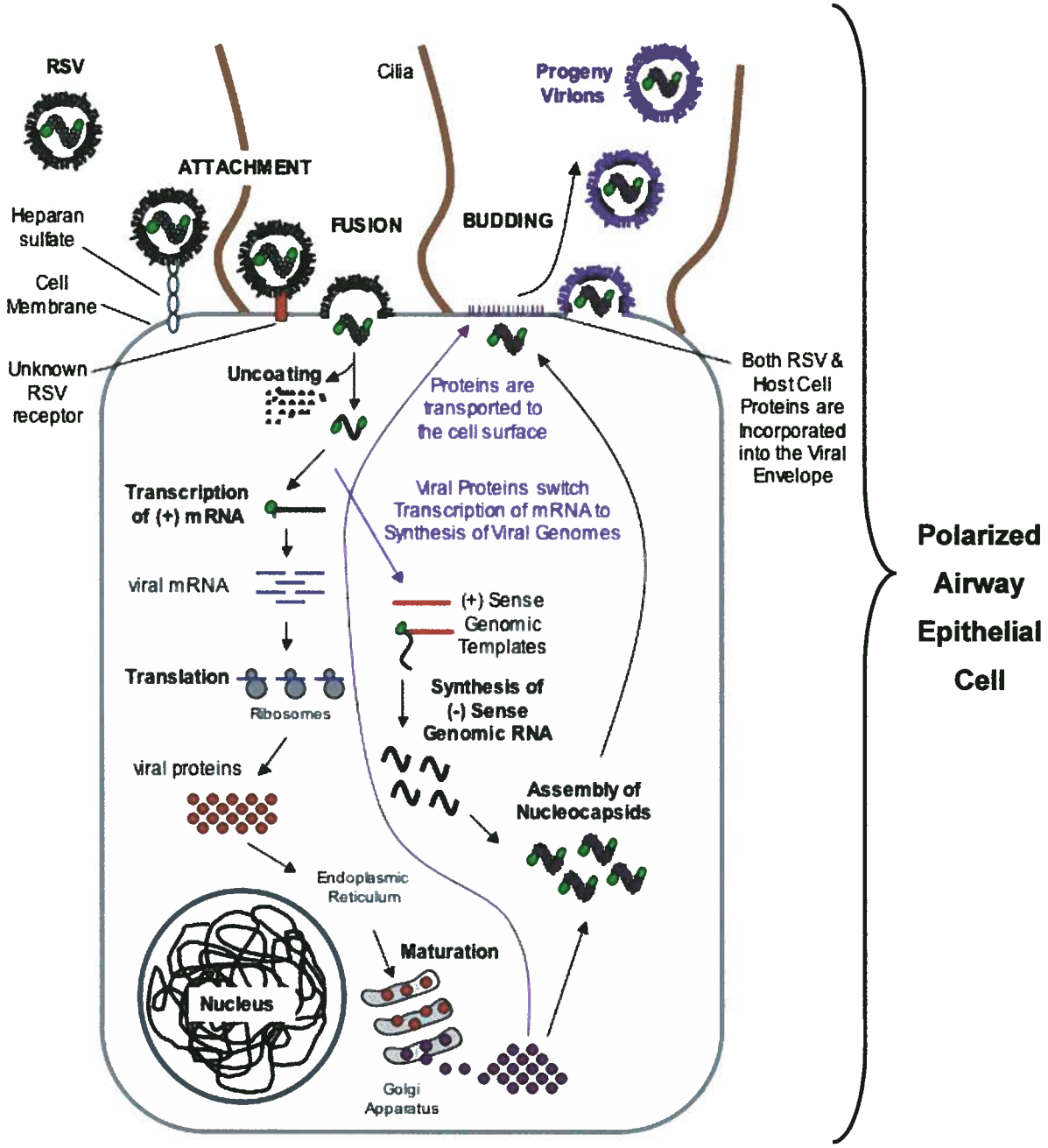


Figure 1.3 RSV Life Cycle RSV binds to heparan sulfate and then undergoes fusion with the host cell plasma membrane via interactions between the F protein and the unidentified RSV receptor. The viral nucleocapsid is then injected into the cytoplasm, uncoats, and becomes transcritionally active. Viral proteins are subsequently synthesized until large amounts of the M2-2 protein are present. This results in switching from transcription of viral mRNA's to the production of full-length positive sense templates which are then transcribed into negative sense genomic RNA. The N and P proteins associated with the genomic RNA and in coordination with the M protein render it inactive. The N, P and viral polymerase are then packaged into a nucleocapsid. The M protein chaperones the nucleocapsid to lipid rafts on the host cell surface that have become enriched with G, F and SH protein. Subsequently, the virion buds from the cell membrane and proceeds to infect nearby or neighboring cells.

1.1.6 Host Response to RSV Infection

In response to infection, airway epithelial cells produce reactive oxygen species, such as nitric oxide, and release several chemokines and cytokines. The chemokines released include RANTES (Regulated on Activation, Normal T Expressed and Secreted), interleukin (IL)-8, monocyte chemotactic protein (MCP)-1, and macrophage inflammatory protein (MIP)-1 alpha. Cytokines released include tumor necrosis factor (TNF)-alpha, IL-1 alpha, IL-6, and IL-10 (90-93). Type I interferons (IFN-alpha/beta) are also expressed, however, their expression is down regulated by the RSV NS-1 and 2 proteins (70). Together, these chemokines and cytokines promote the recruitment and activation of T cells, monocytes, macrophages, eosinophils, and neutrophils to the site of infection (94-97). In addition, IFN-alpha/beta enhance antigen presentation by the airway epithelial cells and attract natural killer (NK) cells (98). At the site of infection, NK cells produce IFN-gamma and kill infected cells (94).

In parallel, dendritic cells (DC) and macrophages initiate an adaptive immune response (99, 100). DC and macrophages engulf RSV and migrate to regional lymph nodes and produce the cytokines IFN-gamma, IL-12, and IL-10 (101). In the lymph nodes, these cells present viral antigens to naïve CD4+ T helper cells, which, depending on the local cytokine environment, differentiate into either T helper (Th)-1 or -2 cells (102-104).

A predominant Th1 differentiation results in cell-mediated immunity, which is required for an effective anti-viral response and is thought to limit RSV infection to the upper respiratory tract (103). Th1 cells release IFN-gamma, IL-2 and IL-12 (94) and, through the actions of IFN-gamma and TNF-alpha, promote activation of CD8+ cytotoxic T cells towards RSV infected cells. Further, Th1 cells also interact with B cells to induce the production of neutralizing IgG2a antibodies (94). In contrast, Th2 differentiation leads to an allergic immune response that is required for the clearance of extracellular pathogens, and is thought to result in severe RSV infection (105). Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 (103). These cytokines promote

eosinophil and mast cell recruitment, and induce B cells to produce RSV-specific IgE and IgG1 antibodies (106). Importantly, Th1 and Th2 responses negatively regulate each other, thus the ratios of IL-4 to IFN-gamma, and IL-10 to IL-12, are used as indicators of the predominant immune response.

In clinical studies, stimulated peripheral blood mononuclear cells (PBMC) isolated from children with RSV bronchiolitis have an increase or Th2 skewed IL-4/IFN-gamma cytokine ratio relative to healthy controls (107). Prospective studies showed children that develop severe RSV infection have a Th2 skewed IL-4/IFN-gamma and IL-10/IL-12 cytokine ratio in their nasal lavage fluids relative to children that developed a RSV upper respiratory tract infection (URTI) alone (108). Moreover, when PBMC's were isolated and stimulated from the same children, they showed an elevated/Th2 skewed IL-4/IFN-gamma ratio relative to children with RSV URTI (108).

Thus, in summary, RSV is a serious disease in humans with complex pathogenesis. To date no effective vaccines have been developed and the available prophylactic approaches are only 50% effective. Consequently, new approaches are needed to address RSV and diminish its disease burden. One approach, as discussed in Chapter 2, may be direct inhibition of viral invasion via mPEG-modification (PEGylation) of either the host cell surface or virus itself.

1.2 METHOXYPOLY(ETHYLENE GLYCOL) [mPEG] AND PEGYLATION

1.2.1 Historical Background

Methoxypoly(ethylene glycol) [mPEG] is a non-toxic, FDA approved polymer of low immunogenicity. mPEG can be grafted to proteins in a process referred to as PEGylation. In the 1970's, mPEG was first grafted to bovine albumin and catalase, and these mPEG-protein conjugates were shown to have decreased immunogenicity and a prolonged plasma half-life in rabbits and mice (109, 110). This started the field of protein-PEGylation and, currently, several PEGylated proteins are in use as pharmaceutical therapies (see section 1.2.5).

Based on this technology, mPEG-modification of red blood cells (RBC) was evaluated by Dr. M. Scott and was shown to effectively camouflage cell surface antigens while maintaining normal RBC function and *in vivo* survival (111, 112). Subsequently, mPEG has been used to modify white blood cells (WBC), platelets, viruses, and virus host cells (113-116). Importantly, mPEG modification of the virus or viral host cells has been shown to prevent infection by several viruses. Thus, as outlined in the following chapter, the goal of this work is to extend these findings and evaluate the efficacy of mPEG grafting to viruses or their host cells, specifically in the context of RSV infection.

1.2.2 mPEG Chemistry

mPEG is a derivative of polyethylene glycol, which has the following chemical structure $HO\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH$. As both hydroxyl groups at either end of the polymer are potentially reactive, one group is modified with a methyl group to produce methoxypoly(ethylene glycol) with the formula $CH_3O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH$ (117). For protein modification, the remaining hydroxyl group is substituted with a functional group that is

reactive towards lysine residues. Several linker or activation chemistries have been developed and the linker chemistries used in this thesis include cyanuric chloride, succinimidyl carbonate, and succinimidyl valerate (118) to produce cyanuric chloride mPEG (CmPEG), succinimidyl carbonate mPEG (SCmPEG), and succinimidyl valerate mPEG (SVAmPEG), respectively(see Figures 1.4-1.6). mPEG polymers can be synthesized as linear or branched molecules and linear polymers are commercially available in lengths ranging from 2-20 kDa (119).

For mPEG-modification of proteins, peak grafting rates occur when activated mPEG is reacted with lysine residues at moderate temperatures (~25°C) and high pH (>10). As shown in Figure 1.4(a), at high pH the negatively charged form of lysine predominates and in this ionization state the epsilon-amino group is neutrally charged. For mPEG grafting, this neutrally charged amino group acts as a nucleophile and undergoes a nucleophilic displacement reaction with the carbonyl carbon of mPEG. This results in the formation of a new covalent bond between mPEG and the protein, and the release of the linker molecule into solution (see Figures 1.5-1.7).

Unfortunately, highly basic conditions are not biologically compatible and may denature proteins, or in the case of cells or viruses disrupt the integrity of the outer membrane. Thus for most protein or cell modification a pH range from 7.8 – 8.0 is used. As shown in Figure 1.4(b), at this pH, lysine exists predominantly in the 1+ cationic state, however, it is in equilibrium with a small amount of the –1 anion. This anion is capable of undergoing nucleophilic displacement with activated mPEG and is replenished by a shift in the equilibrium as lysine reacts with mPEG.

To further illustrate the reaction of mPEG with lysine residues, the reaction schemes for several mPEG species are outlined in Figures 1.5-1.7. It is important to note that while both SCmPEG and SVAmPEG undergo nucleophilic displacement that produces the linker chemistry as a leaving group, cyanuric chloride does not undergo the same reaction. Instead, the nucleophilic displacement occurs between the carbon in the fourth position of the triazine ring

and the epsilon amino group of lysine residue. Thus, chlorine is the leaving group and the linker chemistry ring structure remains intact between the mPEG chain and the lysine residue (120). Further, despite the naming of cyanuric chloride mPEG, this species does not contain cyanide nor is cyanide created during its reaction with lysine or N-terminal amino groups.

As illustrated in these figures, there is a competing hydrolysis reaction during PEGylation in which water acts as a nucleophile competing with lysine in the nucleophilic displacement. Under basic conditions (pH 8.0) at room temperature, the hydrolysis half-life for SC and SVAmPEG are 20.4 and 33.6 minutes, respectively (119), while for CmPEG the half-life has not been determined but is approximately 10 minutes (120). It is important to note that pH plays a significant role in determining the extent of hydrolysis; specifically, as the pH is lowered by one unit from pH 8 to 7, the rate of hydrolysis triples (121).

Theoretically, activated mPEG species can graft to any appropriately charged amino group. Several experimental studies, however, have shown that lysine residues are the primary sites of mPEG grafting (122-124). This is exemplified by studies of PEGylated IFN-alpha in which interferon was modified with a 40 kDa branched mPEG. In these studies, the N-terminal cysteine residue was not PEGylated, while according to mass spectrometry and amino acid sequencing lysine residues 31, 70, 83, 121, and 134 were the sites of mPEG grafting (122).

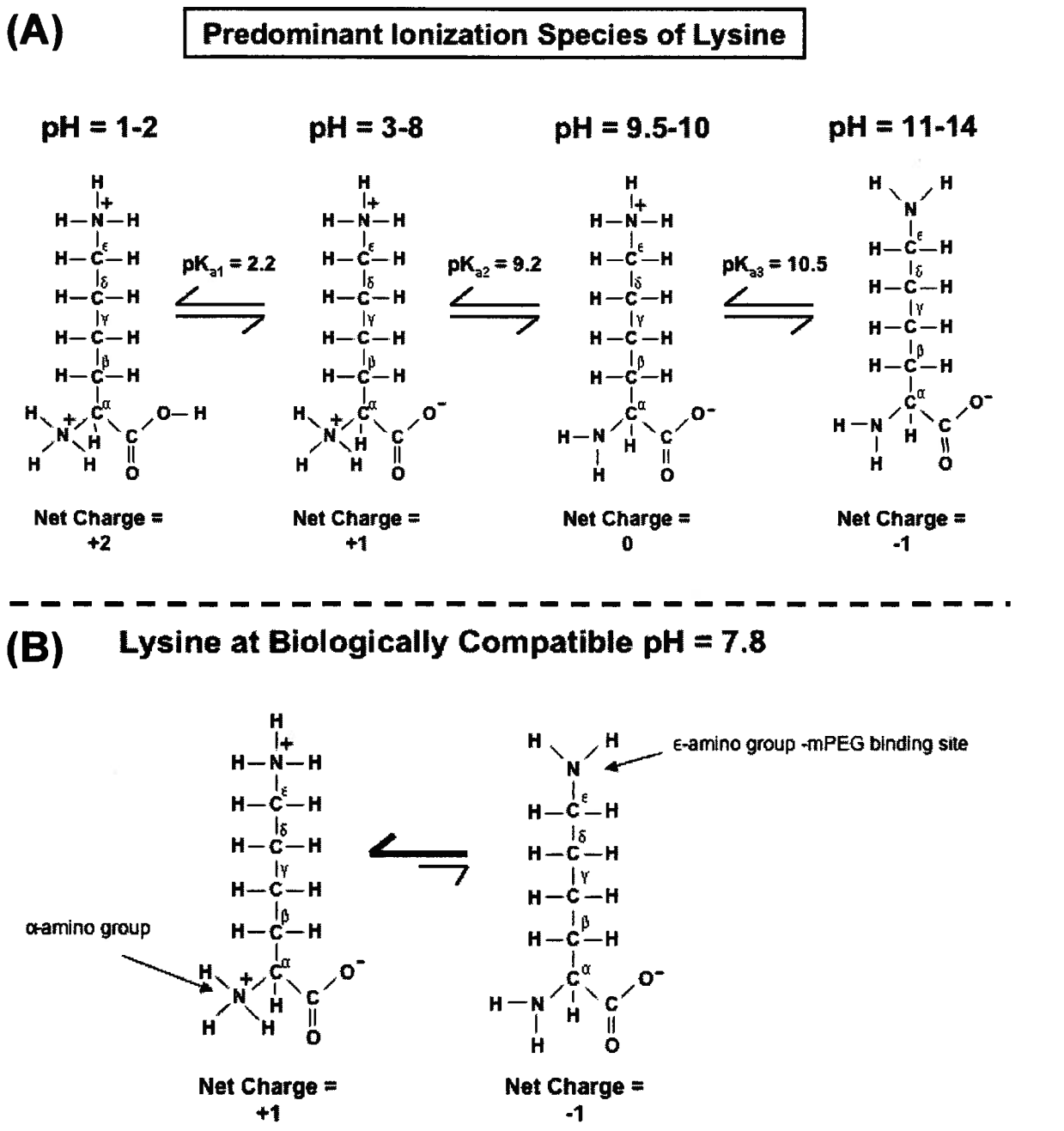


Figure 1.4. Structure of Lysine. **(A)** Lysine exists in equilibrium with all of its ionization states, however at a specific pH one ionization state will predominant. **(B)** For mPEG-modification of proteins, the optimal pH is very basic, however this is not biologically compatible, thus pH 7.8 is used. At this pH the positively charged form is predominant, however a small amount of the negatively charged species shown on the right is capable of reacting with activated mPEG. Importantly, as this species is depleted in grafting reactions it is regenerated due to a shift in the equilibrium. Thus, lysine amenable to reacting with mPEG will continue to be generated. Modified from Lehninger AL, (125).

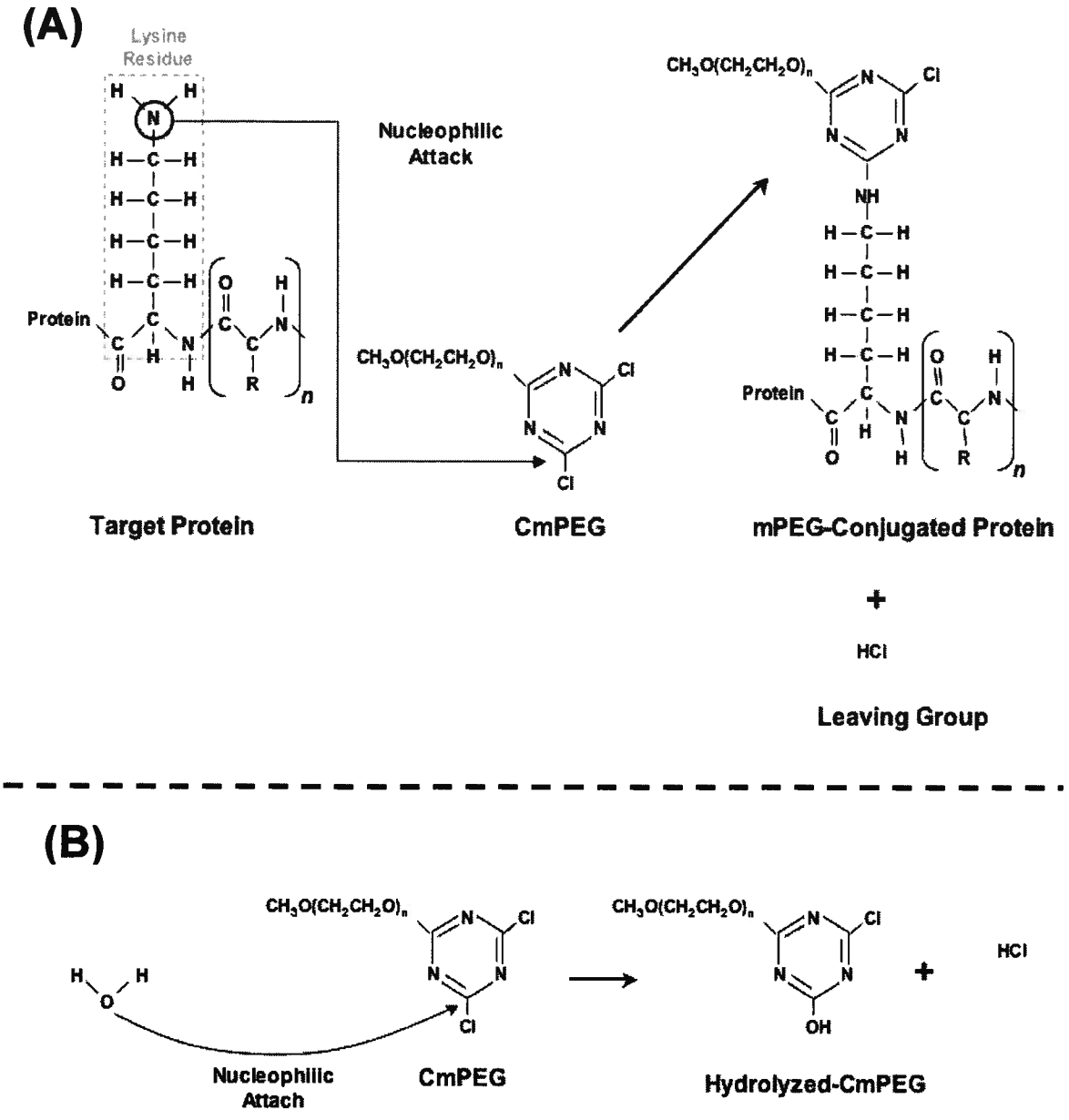
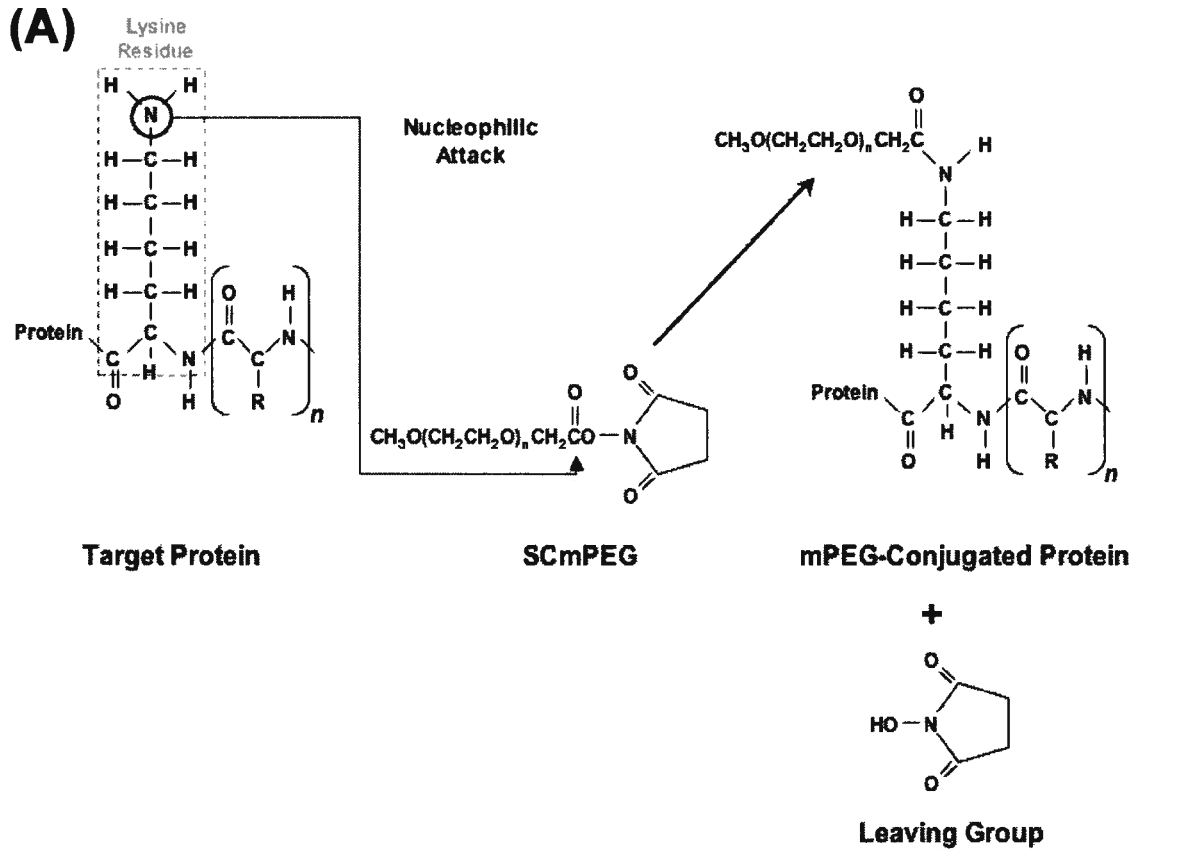


Figure 1.5. CmPEG – Lysine Reaction and Hydrolysis **(A)** CmPEG-lysine residue reaction. The ε-amine group in the lysine residue acts as a nucleophile and undergoes nucleophilic substitution with the chlorine atom in the 4th position on the triazine ring of CmPEG. This results in the formation of a new covalent bond between the triazine ring of CmPEG and the ε-amine group in the lysine residue. **(B)** Hydrolysis of CmPEG. During mPEG modification of proteins, water competes with lysine in a hydrolysis reaction. Note: Unlike other linker chemistries, CmPEG retains its linker chemistry when grafted to a protein.



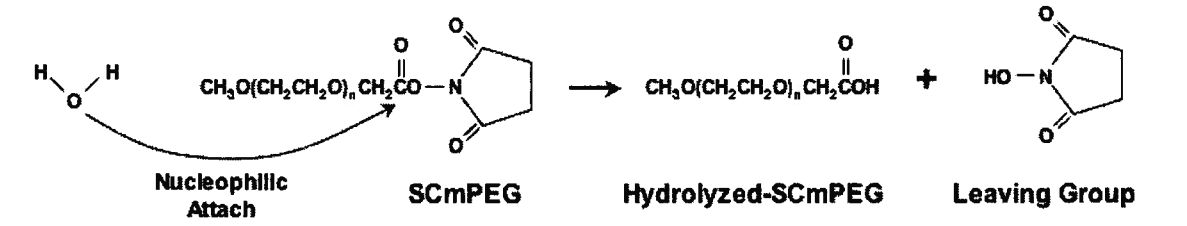


Figure 1.6. SCmPEG – Lysine Reaction and Hydrolysis. (A) SCmPEG-lysine residue reaction. The ε-amino group in the lysine residue acts as a nucleophile and undergoes nucleophilic substitution with the carbonyl atom in SCmPEG. This results in the formation of a new covalent bond between the carbonyl atom and the ε-amine group in the lysine residue. **(B)** Hydrolysis of SCmPEG. During mPEG modification of proteins, water competes with lysine in a hydrolysis reaction. Note: During the mPEG reaction with lysine, the linker chemistry is released.

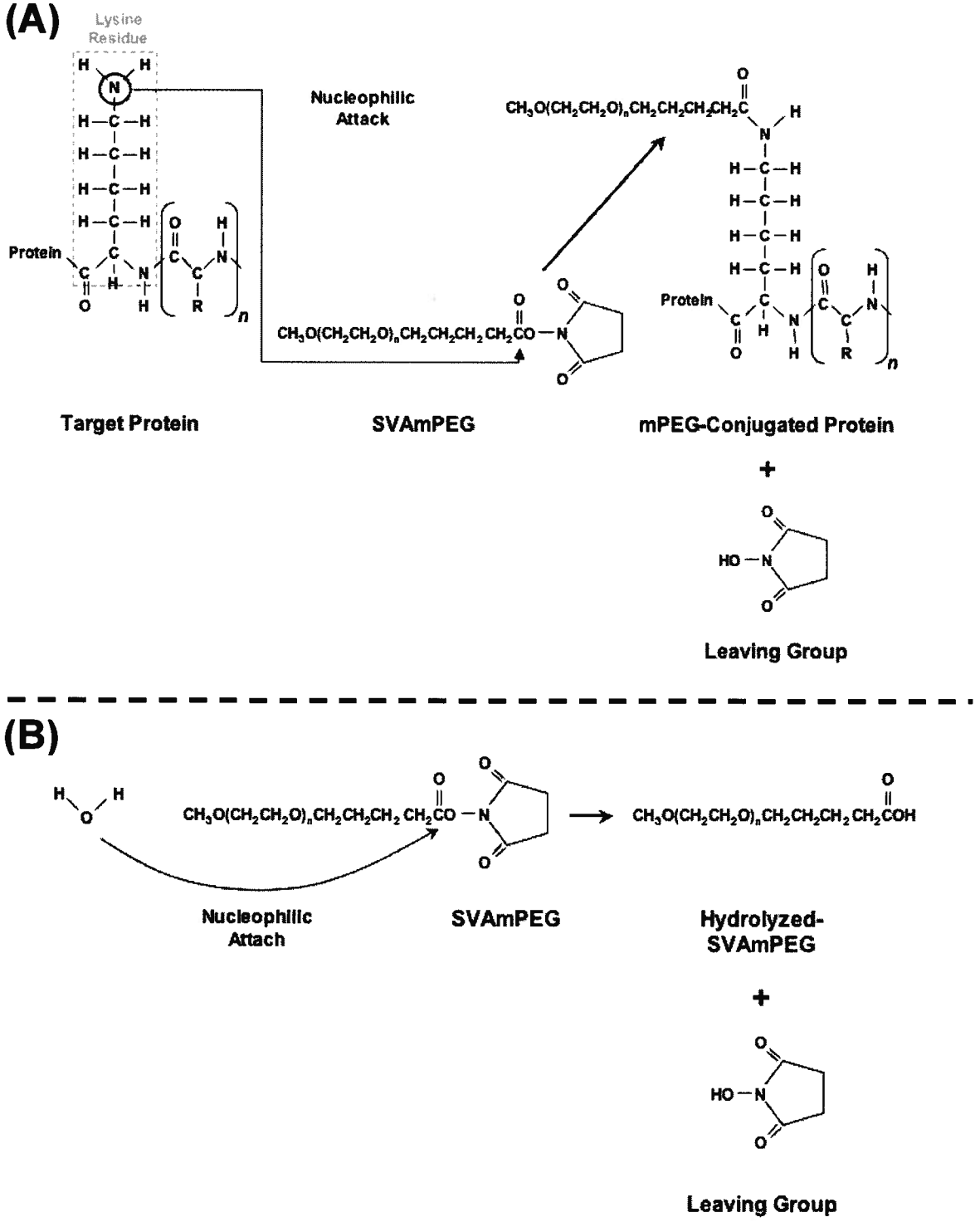


Figure 1.7. SVAmPEG – Lysine Reaction and Hydrolysis. **(A)** SVAmPEG-lysine residue reaction. The ε-amino group in the lysine residue acts as a nucleophile and undergoes nucleophilic substitution with the carbonyl atom in SVAmPEG. This results in the formation of a new covalent bond between the carbonyl atom and the ε-amine group in the lysine residue. **(B)** Hydrolysis of SVAmPEG. During mPEG modification of proteins, water competes with lysine in a hydrolysis reaction. Note: During the mPEG reaction with lysine, the linker chemistry is released.

Table 1.1. Hydrolysis Half-Lives of CmPEG, SCmPEG, and SVAmPEG
Half-lives are determined at 25 °C in pH 8.0 buffer (119). * The exact half-life of CmPEG has not been determined, but is an estimate based on results by Johnson *et al.*(120).

Linker Chemistry / mPEG Species	Hydrolysis Half-Life (Room Temperature, pH 8.0)
CmPEG	~10 minutes*
SCmPEG	20.4 minutes
SVAmPEG	33.6 minutes

1.2.3 Physical Properties of mPEG and mPEG Layers

mPEG polymers are highly flexible molecules with free rotation every 4-5 ethoxy units. Thus, when an mPEG polymer is grafted to surface or a protein, the mPEG chain develops a radius of gyration (R_g) that is approximately equal to the length of the polymer (126) (see Figure 1.8(a)). Additionally, mPEG is very hydroscopic and binds 2-3 water molecules per ethylene oxide unit (127). Thus, as shown in Figure 1.8, given both its radius of gyration and increased volume due to hydration, one mPEG polymer acts as if it were 5-10 times as large as a rigid polymer of comparable size (117). When grafted to a surface, the mPEG polymer consequently occupies a large space or volume above the surface and excludes other proteins and macromolecules (112, 113, 126). In this fashion, the mPEG polymer will protect both the protein it is bound to and proteins in the surrounding area.

Considering the 3-dimensional structure of mPEG bound to an artificial (plastic) planar surface, when mPEG is grafted at low densities the polymers exist as separate coils and form mushroom-shaped structures (128, 129) (see Figure 1.9). As shown in Figure 1.10, when mPEG is grafted to a cell surface, this mushroom shaped mPEG conformation produces a zone of exclusion. In this zone, the region very close to the cell surface has low mPEG density represented by the covalent bond between mPEG and the surface. As we move outwards from the surface, the mPEG density increases as the bulk of the polymer is encountered; then moving

farther out mPEG density again decreases (see Figure 1.10)(128-130). Importantly, this mPEG gradient results in a gel-like zone above the surface capable of excluding large proteins and particles such as antibodies, cells, and, potentially, virus particles (130).

When mPEG is grafted at high densities, mPEG chains are forced into close proximity and, therefore, have limited rotation (131, 132). Consequently, the mPEG chains exist in a relatively straight conformation and produce a brush-regime or border (see Figure 1.9). The mPEG chains in this regime interact via van de Waals forces and interchain hydrogen bonding resulting in chain entanglement. This in turn produces a more dense and uniform mPEG layer (132). This border similarly excludes large molecules and particles, however it will provide more effective protection than a low-density barrier. Although this mPEG-rich zone excludes large proteins and particles, small molecules and nutrients, such as glucose, water, and oxygen, are still able to pass through the mPEG layer (111, 112, 126). In this fashion, the mPEG-layer acts as molecular sieve excluding large molecules while allowing small molecules to pass through.

Depending on the surface protein distribution and polymer grafting density, it is likely that both forms of mPEG boundaries will exist when mPEG is bound to a surface. However, it is unlikely that mPEG can be grafted at densities that will produce a brush border covering the entire surface of a biological membrane. Furthermore, surface protein topography will also have significant effects. For example, large proteins will likely extend above the mPEG-rich zone making them susceptible to interactions with other proteins and cells. In contrast, small proteins will be hidden within the mPEG-rich zone and will not be able to interact with other proteins or particles that approach the surface. Additionally, polymer length will also significantly affect the height of the mPEG layer. mPEG polymers used in this thesis range in size from 2 to 20 kDa. When these polymers are grafted to a surface, they extend from 6 nm up to 50 nm (see Figure 1.11) from the surface (133). Thus, surface topography, polymer length, and grafting density

will all interact to determine the extent to which the mPEG layer prevents interactions between surface proteins and proteins or cells in solution.

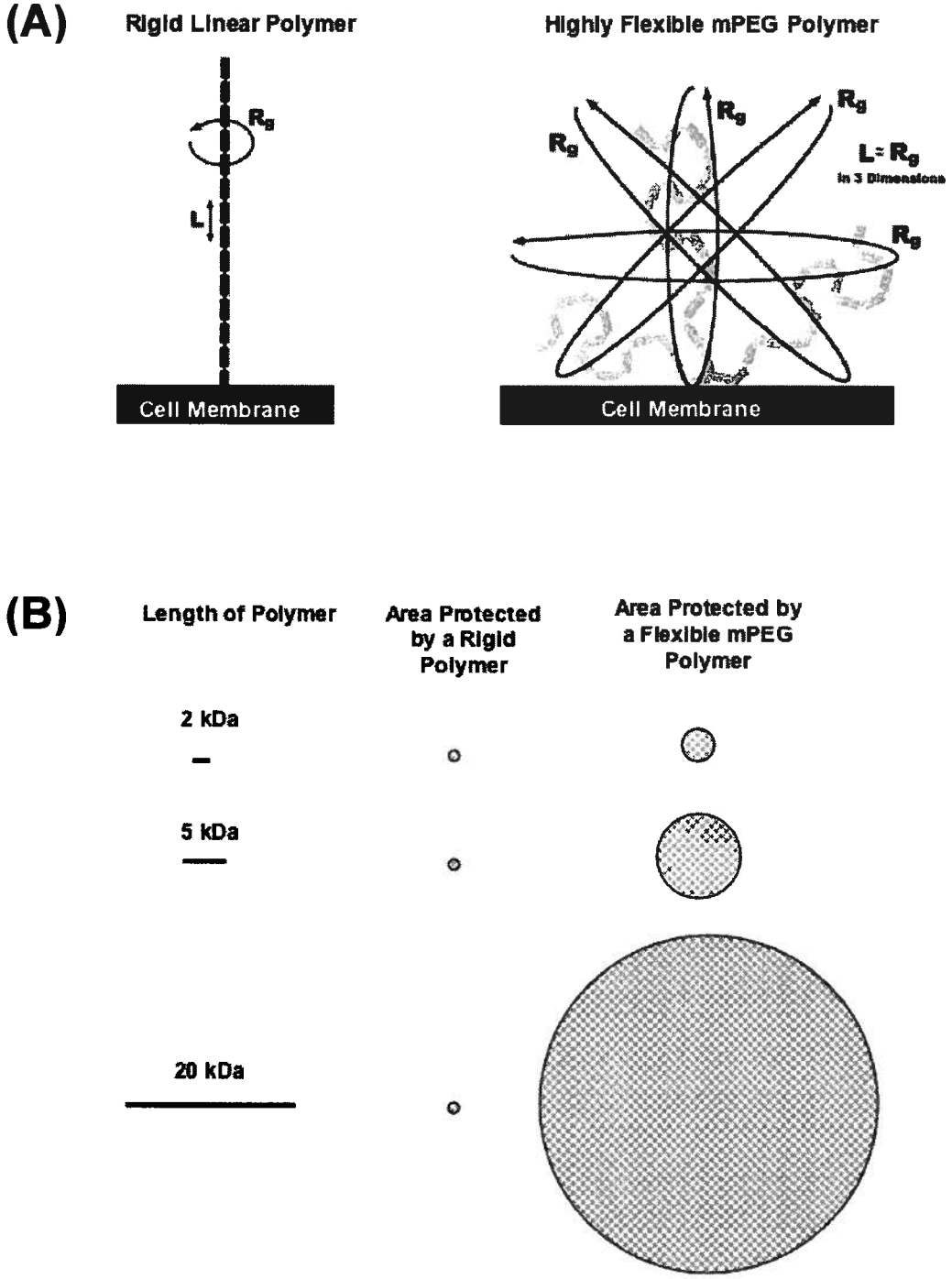


Figure 1.8. Radius of Gyration and Area of Protection. **(A)** Rigid linear polymers have a very small radius of gyration. In contrast, flexible mPEG polymers have a radius of gyration that is approximately equal to the chain length. **(B)** The large radius of gyration of flexible mPEG polymers results in a much larger area of protection than rigid polymers. Modified from Chen and Scott (2006)(134), and McCoy and Scott (2005) (135).

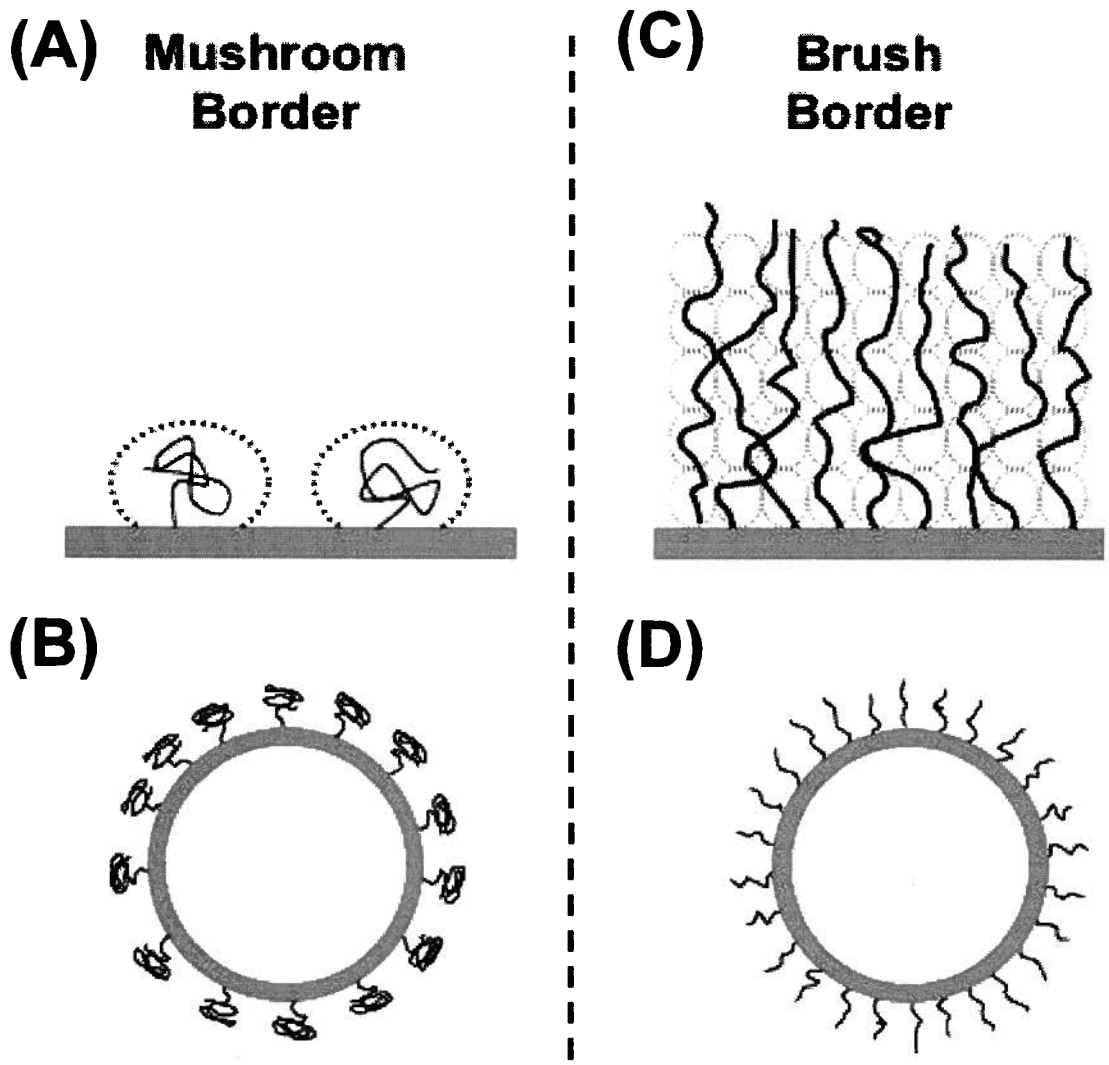


Figure 1.9. Mushroom and Brush Borders. **(A)** Mushroom Border visualized close to the grafted surface. **(B)** Mushroom Border visualized at a distance from the grafted particle. At low grafting density, mPEG chains coil and have a mushroom shaped area of protection. Mushroom borders usually form on cell surfaces. **(C)** Brush Border visualized close to the surface of the grafted particle. **(D)** Brush Border visualized at a distance from the grafted particle. At high grafting density, mPEG chains remain in a relatively straight chain conformation and form a dense packed layer. Brush borders are usually formed on artificial (*i.e.* plastic) surfaces. Brush borders are difficult to generate on cells or biological surfaces as proteins have a heterogeneous distribution on the cell membrane. Modeled from deGennes, 1980 (128) and Allen, 2002 (129).

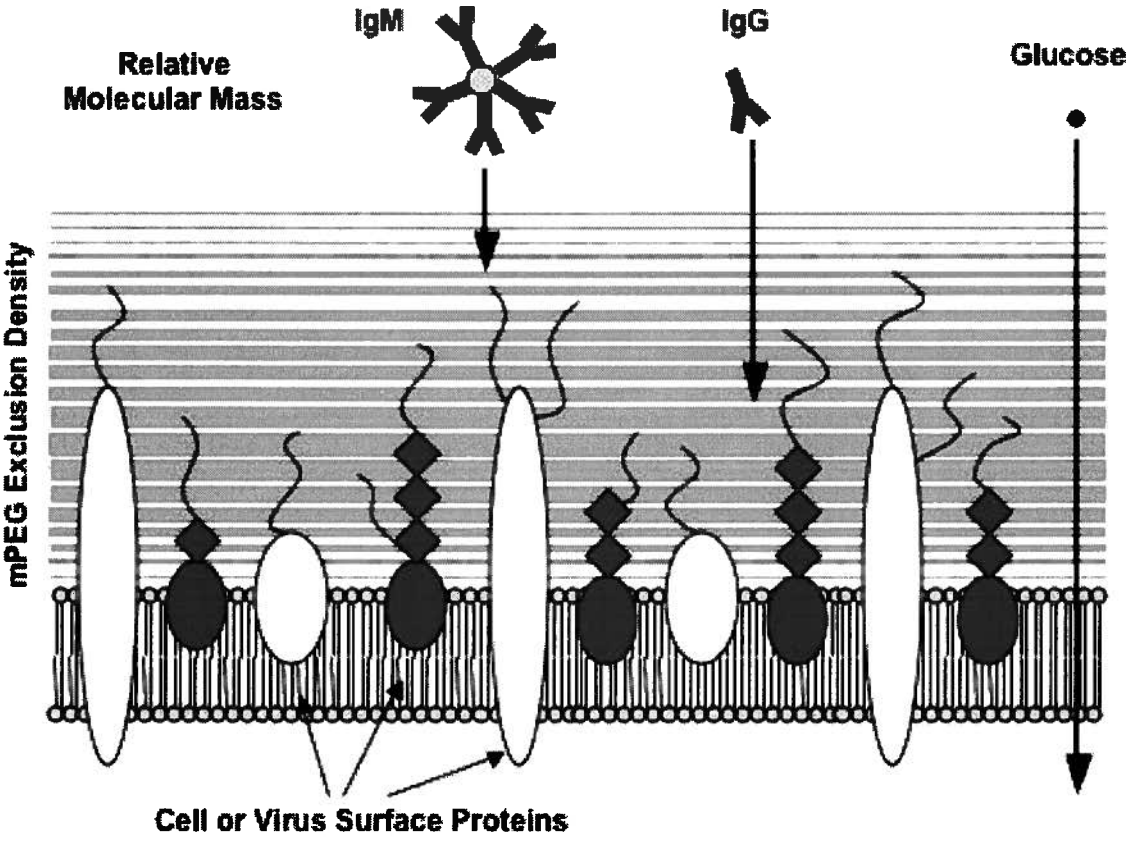


Figure 1.10. mPEG Zone of Exclusion. At the cell or virus surface mPEG grafting creates a zone of exclusion indicated by horizontal gray bars. The density of this zone is variable with maximal density midway from the cell surface to the tip of the longest protein. This mPEG zone acts as a molecular sieve allowing small molecules (*i.e.* water, glucose and salts) to pass through uninhibited. In contrast, larger molecules (*i.e.* IgG) cannot penetrate the zone, therefore preventing their interaction with surface proteins. Note: This figure does not adequately address the variation in mPEG density due to non-uniform protein distribution. Modified from Bradley *et al.,* (130).

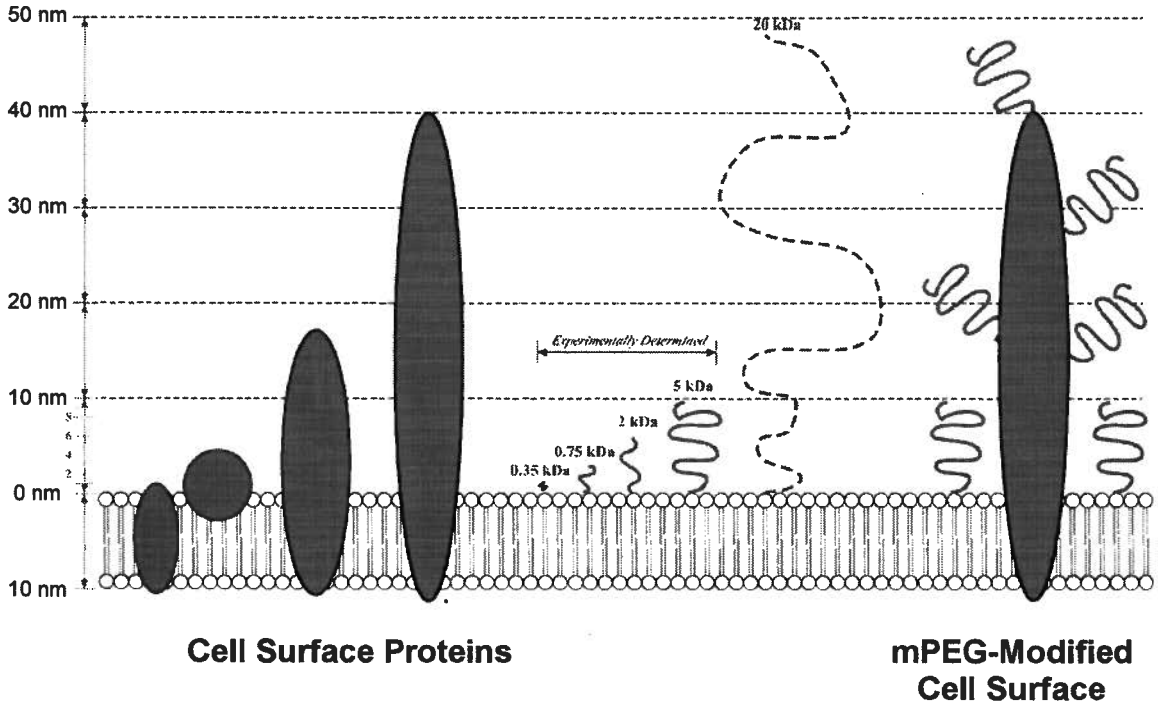


Figure 1.11. Length of mPEG Polymers Grafted to a Biological Surface. Illustrated are the lengths of mPEG chains bound to a cell or virus surface. The length of 0.35, 0.75, 2, and 5 kDa polymers grafted to a surface have been experimentally determined, (133) while the length of 20 kDa is theoretical. Figure courtesy of Dr. M. Scott.

1.2.4 mPEG Toxicity and Immunogenicity

mPEG is FDA approved for parental use, topical applications, and as a constituent of nasal sprays, foods, and cosmetics (136). Upon oral administration, mPEG's less than 30 kDa in length are primarily excreted in the urine, while large mPEG polymers (>30 kDa) are excreted in the feces (137). mPEG has little to no toxicity when administered orally or parentally. Toxicity arises when large doses of mPEG are administered orally or when low molecular weight mPEG's are administered either topically or via intravenous injection (138). The toxicity noted with large oral doses is due to increased gastrointestinal flow producing diarrhea. For low molecular weight polymers (<400 Da), toxicity is the result of mPEG oxidization by alcohol dehydrogenase to produce toxic acid metabolites. Hence, longer mPEG species (>1000 Da) that are not readily oxidized by alcohol dehydrogenase are used in most drug formulations (138). Additionally, mPEG has also been shown to have no adverse reproductive effects, and is not carcinogenic when administered orally, or via intraperitoneal, intravenous, and subcutaneous injection to various experimental animals (138).

Despite almost 30 years of research and clinical use that suggests mPEG is non-immunogenic, there are some reports of anti-mPEG antibodies in humans (139-141), (142). In the 1980's, antibodies against mPEG were raised in a small proportion of rabbits immunized with PEGylated ragweed or superoxide dismutase in combination with Freud's complete adjuvant (139). The following year the same authors evaluated the level of anti-mPEG antibodies in patients treated with PEGylated ragweed or bee venom at regular intervals over 1-2 years. They reported that after the first treatment, 50% of patients developed anti-mPEG antibodies. However, after subsequent treatments, the percentage of patients with antibodies declined to 28.5% over two years (140). Further, the antibody response was weak (produced low antibody titers) and consisted of IgM antibodies. Thus, given this limited antibody response and

absence of an increase in antibodies with subsequent mPEG administration, the authors concluded that the antibody response was of little or no clinical significance (140).

More recent studies have shown that with the administration of PEGylated urate oxidase and PEGylated-asparaginase, anti-mPEG antibodies are pre-existing in a subset of patients and are associated with reduced drug efficacy (141, 142). Despite this subset of patients showing pre-existing mPEG antibodies, most patients did not have mPEG-antibodies and the mPEG-protein conjugates were therapeutic and showed increased plasma half-life (141, 142). Thus while it appears that a subset of patients develop anti-mPEG antibodies, in the majority of patients and with the vast majority of mPEG-protein conjugates, mPEG does not induce a robust immune response and is largely non-immunogenic. Indeed, these studies suggest that the "foreign" protein may act as the hapten promoting an immune response that may facilitate the production of anti-mPEG antibodies.

Animal studies have demonstrated that repeated administration of PEGylated viruses or cells induces little or no antibody response against mPEG. For example, the administration of PEGylated adenovirus vectors has been evaluated as a gene therapy in mice (143, 144). Surprisingly, in these studies, repeated administration of mPEG-modified adenovirus resulted in decreased gene transfection suggesting that mice may be developing anti-PEG antibodies. However, changing the linker chemistry restored transfection efficiency showing that antibodies were not being developed against the mPEG polymer itself, but rather against a new epitope created by the linker chemistry bound to a viral surface protein (144). In the context of mPEG-modified red blood cells (RBC), the *in vivo* survival of hypertransfused mPEG-modified RBC has similarly been evaluated (113, 145). When mice were transfused up to 33 times with mPEG-modified RBC, the RBC showed normal survival and circulation time, indicating that no anti-mPEG antibody response had developed. Furthermore, mice exhibited normal behaviour, physical activity, and viability, suggesting that the transfusion of PEGylated RBC did not have

any associated toxicity and was well tolerated (113, 145). Thus, while humans show some anti-mPEG antibody response, this response appears to be minimal, and have little effect on therapeutic efficacy. Similarly, in animals, PEGylated cells and vectors may not be completely inert. However, even when given repeatedly, no antibody response develops against the mPEG polymer.

1.2.5 PEGylated-Protein Therapeutics

The first PEGylated proteins were bovine albumin and catalase (109, 110). Following the demonstration that these proteins had greatly reduced immunogenicity and prolonged circulating half-life in mice and rabbits, PEGylated adenosine deaminase (PEG-ADA) was evaluated as a treatment for adenosine deaminase deficiency in humans (146). Subsequently, PEG-ADA was brought to market as Adagen (146), and since its introduction, several PEG-conjugates have been developed to treat a variety of conditions. These conditions range from treatment of cancer and hepatitis C infection, to replacement of blood coagulation factors and hormones (122, 124, 147-149). Several PEGylated proteins are currently in use as therapeutics including asparaginase, bilirubin oxidase, IFN-alpha, beta and gamma, IL-2, methionase, superoxide dismutase, thrombin, and recombinant coagulation factor VIII (122, 147, 149-158). Further, mPEG-protein conjugates continue to be developed as is evident by recent reports of mPEG-gemcitabine as an anti-cancer therapy and mPEG-proline-3 gastric inhibitory polypeptide as a treatment for diet induced diabetes (159, 160). At present, while mPEG continues to be used as a means of improving drug efficacy, the field of cell and organ PEGylation is expanding and represents the next step in the evolution of this technology.

1.2.6 PEGylated Blood Cells

1.2.6.1 PEGylated Red Blood Cells

Pioneering work on PEGylation of cell surface proteins on intact cells was originally conducted with red blood cells (RBC). In these studies, Scott *et al.,* showed that mPEG grafting to RBC surface proteins resulted in a loss of antibody mediated aggregation of the ABO blood group antigens (e.g., A) and non-ABO antigen (e.g., RhD) (see Figure 1.12(a)) (111, 113, 130, 145, 161, 162). Similarly, these studies showed that mPEG-modified sheep RBC were not readily engulfed by human monocytic cells suggesting that mPEG modification disrupted immune recognition (see Figure 1.12(b)). Thus to evaluate the immunogenicity of mPEG-modified RBCs, mPEG-modified sheep RBC were administered to mice and shown to induce a significantly weaker antibody response relative to unmodified controls (111, 112) (see Figure 1.12(c)). Further, mPEG-modified sheep RBC showed reduced anti-sheep antibody binding when administered to mice that had been previously immunized with sheep RBC (111). Importantly, mPEG modified RBC were shown to have normal morphology and osmotic fragility, and also showed normal *in vivo* survival time (see Figure 1.12(b)) even with repeated transfusions (111, 112).

As it was evident that mPEG was masking cell surface antigens and effectively hiding them from the host immune system, the term "immunocamouflage" was coined. In subsequent studies, mPEG modification was also shown to hide the surface charge on a cell which could potentially inhibit cell-cell interactions. This was examined by studying the electrophoretic mobility of mPEG-modified RBCs. In an electric field, unmodified RBCs were shown to have a mobility rate of -1.18 μm.cm/volt.sec; however, when RBCs were modified with increasing concentrations of benzotriazole carbonate (BTC)-mPEG or N-hydroxysuccinimidyl ester of mPEG proprionic acid (SPAmPEG), electrophoretic mobility diminished as a function of mPEG grafting density and polymer length. Specifically, grafting at high mPEG concentrations with

long 20 kDa polymers resulted in a dramatic decrease in mobility that approached zero. In contrast, grafting with shorter polymers resulted in a decrease in electrophoretic mobility that reached a plateau at approximately -0.85 (~30% reduction) and -0.5 (~60% reduction) μm.cm/volt.sec for 2 and 5 kDa polymers, respectively (163). Thus mPEG modification results in camouflage of both cell surface antigens and the cell surface charge.

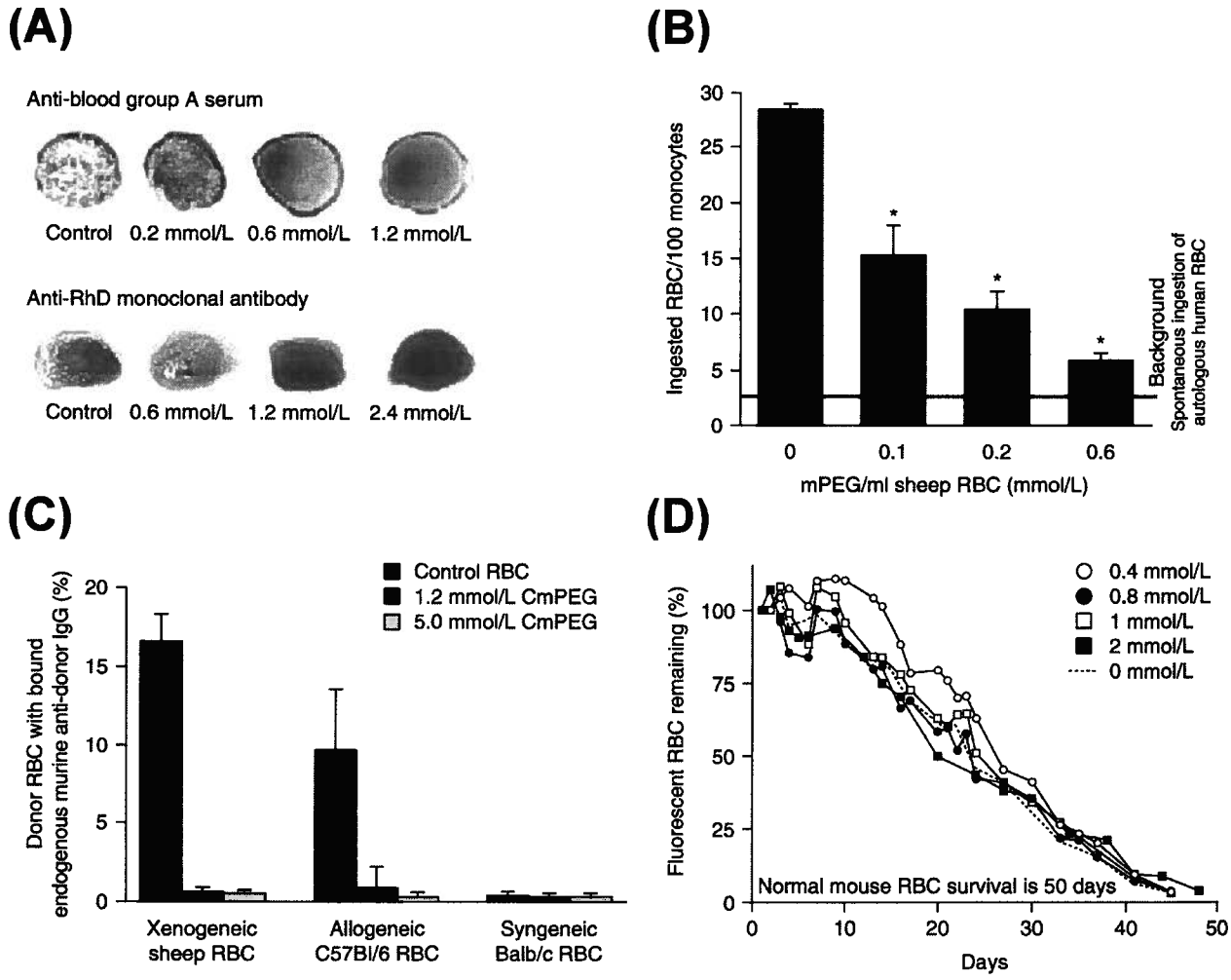


Figure 1.12. Red Blood Cell Immunocamouflage. mPEG grafting to RBC's has been shown to result in: **(A)** type A or RhD+ blood exposed to increased mPEG grafting concentrations and treated with anti-A or anti-RhD antibody. mPEG grafting results in a loss of antibody binding/agglutination of A/B and RhD blood group antigens; **(B)** loss of antigenic recognition necessary for phagocytosis (evident by a decrease in the number of ingested mPEG-modified RBCs); **(C)** loss of immunogenicity in both allogeneic and xenogeneic hosts; **(D)** normal *in vivo* survival time upon transfusion of mPEG-modified RBC. Figure modified from Chen and Scott 2001 (112).

1.2.6.2 PEGylated White Blood Cells and Islets

After successful immunocamouflage of RBC, studies were conducted to determine if PEGylation of white blood cells could prevent immune recognition and graft versus host disease (GVHD). Experiments were performed using mixed lymphocyte reactions with lymphocyte populations from disparate donors. The results of these studies showed that mixtures of unmodified cells resulted in massive cell proliferation, while mPEG-modification of one or both cell populations resulted in a dramatic reduction in this proliferative response. Specifically, grafting of 0.6 mM CmPEG resulted in a 75% decrease in cell proliferation while grafting of 1.2 mM CmPEG decreased proliferation by greater than 95% (114, 164). Furthermore, *in vivo* studies with mice showed that administration of mPEG modified lymphocytes from an allogenic mouse prevented a lymphocyte response in the recipient, and in a model of GVHD with lethally irradiated immunocompromised mice, mPEG-modification of foreign donor lymphocytes was able to abolish the induction of disease (114).

As the mechanism of mPEG protection was thought to result from a loss of cell-cell interactions, flow cytometry studies were subsequently conducted to evaluate the cell surface proteins that were immunocamouflaged. As illustrated in Figure 1.13, the flow cytometry analysis showed that mPEG modification blocks antibody-mediated detection of CD50-CD11a, CD28-CD80, MHC II-TCR/CD3, and CD58-CD2, and suggests that cell surface protein interactions between T cells and antigen-presenting cells (APC) were inhibited by mPEG grafting (112, 164). Thus as these interactions play important roles in adhesion, stimulation, and allorecognition between T cells and APCs, this finding clearly showed that mPEG modification was preventing immune recognition and the subsequent immune response. Further, this finding suggested that as mPEG modification was clearly interrupting T cell-APC cell interactions, mPEG grafting might be able to inhibit other cell-cell or cell-virus interactions.

Consequently, mPEG grafting to and transplantation of pancreatic islets has been evaluated and shows potential. Several studies have shown that mPEG grafting to islets does not significantly affect cell viability or function (112, 165-167). In addition, mPEG-modified pancreatic islets cells were able to engraft and reestablish blood glucose homeostasis in a rat model of diabetes (112). Of interest, the PEGylated islets showed improved function relative to unmodified islets, and this was attributed to mPEG shielding of immunogenic proteins expressed on the cell surface as a result of damage during *ex vivo* processing (112).

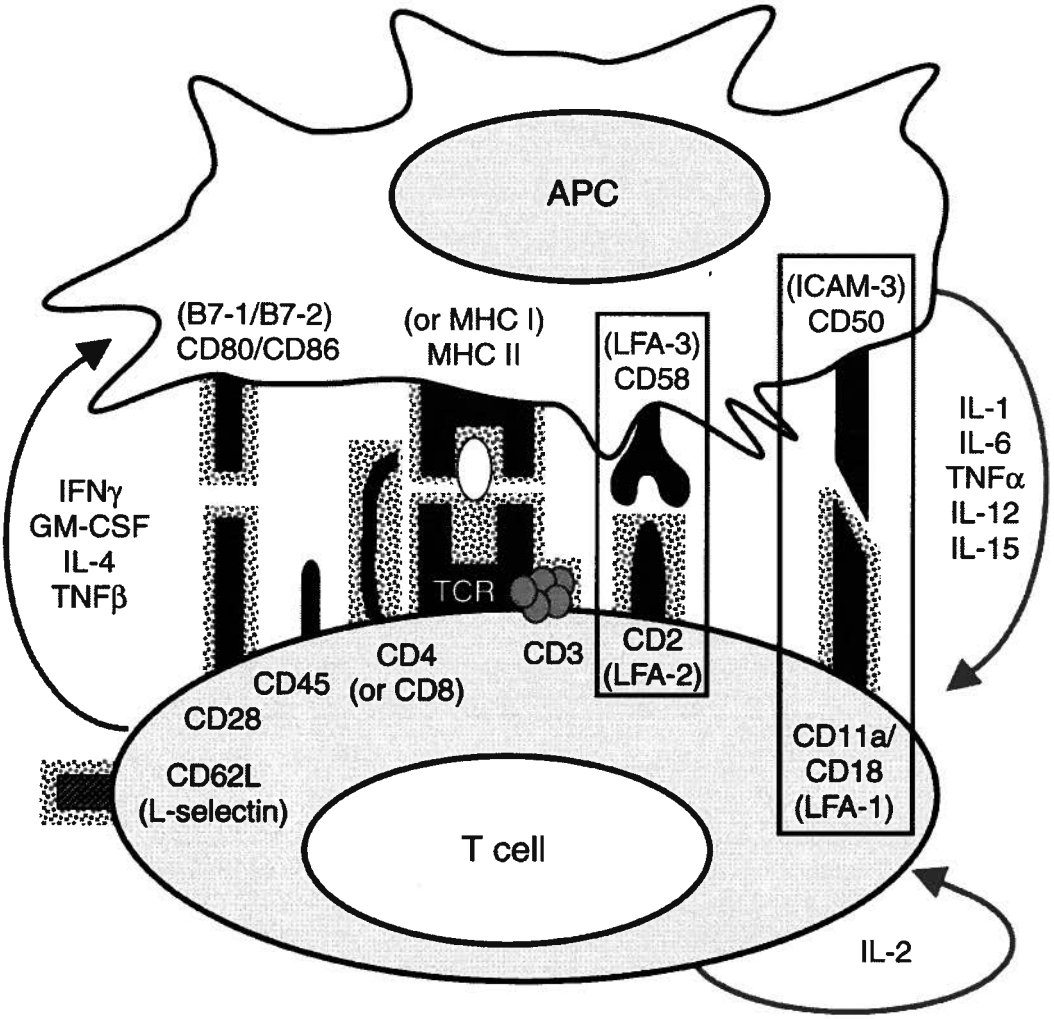


Figure 1.13. mPEG-Modification Prevents Cell Interactions. mPEG-modification of T cells prevents both the adhesion events (CD50:CD11a/CD18 interactions) and activating MHC and co-stimulatory molecule interactions. Shown in the stippled areas are the adhesion, recognition and co-stimulatory molecule interactions blocked by mPEG-modification. Modified from Chen and Scott 2001 (112).

1.2.7 mPEG-Modification of Viruses or Virus Host Cells

1.2.7.1 mPEG-Modified Viruses

To date, direct mPEG modification of virus particles has been evaluated in two contexts: 1) to reduce the immunogenicity of adenovirus gene therapy vectors (115, 143, 168), and 2) to inactivate and inhibit SV40 infection (116, 169, 170). In evaluating mPEG-modification of adenovirus vectors, experiments with 5 kDa Tresyl-mPEG (TmPEG), succinimidyl propionate mPEG (SPAmPEG), and CmPEG have shown that mPEG modification of the virus is restricted to viral surface proteins and that nucleocapsid proteins contained within the virion remain unmodified. *In vivo*, mPEG modification of the adenovirus vector was shown to reduce immunogenicity and clearance of the vector, in turn, enhancing the transduction efficiency (115, 143, 168). Specifically, mPEG modification resulted in decreased binding of neutralizing anti-adenovirus antibodies to the virus, which was the major mechanism of adenovirus clearance. In addition, mPEG modification was shown to interfere with the adenovirus-cell surface receptor interactions and also reduce the surface charge of the virus particle (144, 168). Thus mPEG modification in the context of adenovirus clearly shows similarities to mPEG-modified RBC and WBC. However, it is important to note that mPEG grafting concentrations used to modify adenovirus were dramatically lower than those used in the studies with mPEG-modified SV40 (described below). Furthermore, with mPEG-modification of adenovirus, conditions were optimized to maintain viral infectivity while reducing immunogenicity (168).

Surprisingly, in contrast to PEGylation of adenovirus vectors for gene therapy, research by Scott *et al.* has shown that mPEG-modification of SV40 both inhibits viral invasion and increases immunogenicity (116, 135, 169, 171). As shown in Figure 1.14, in studies with mPEG modified SV40, mPEG modification was shown to inactivate SV40 in a dose-dependent manner, and at high grafting concentrations completely prevented infection (see Figure 1.14). For example, at 15 mM mPEG grafting concentration, no cells became infected with SV40 even

when cells were cultured out to 72 hours post-infection (116, 135). Importantly, grafting of moderate mPEG concentrations resulted in a significant reduction in the number of infected cells, and this reduction prevented the virus from reaching unmodified control levels when followed over 72 hours (116, 135). Furthermore, this mPEG-mediated inactivation was shown with several linker chemistries over a range of mPEG grafting concentrations and polymer lengths (116, 135).

In subsequent *in vivo* experiments, mPEG-inactivated SV40 was administered to mice to determine if mPEG-modification altered the immunogenicity of the virus (169, 171). Examining the antibody response, Mizouni *et al.* found that administration of PEGylated SV40 resulted in an increase in the levels of antibodies against the viral surface protein, viral protein 1 (VP1). This increased immunogenicity was thought to be the result of mPEG-modification enhancing the circulation time of the virus, therefore, prolonging antigen exposure (169, 171).

In summary, these proof of concept experiments show that mPEG-modification may be a novel method of viral inactivation. Therefore, the experiments conducted within this thesis aim to extend these findings and evaluate if mPEG-modification can be used to inactivate RSV.

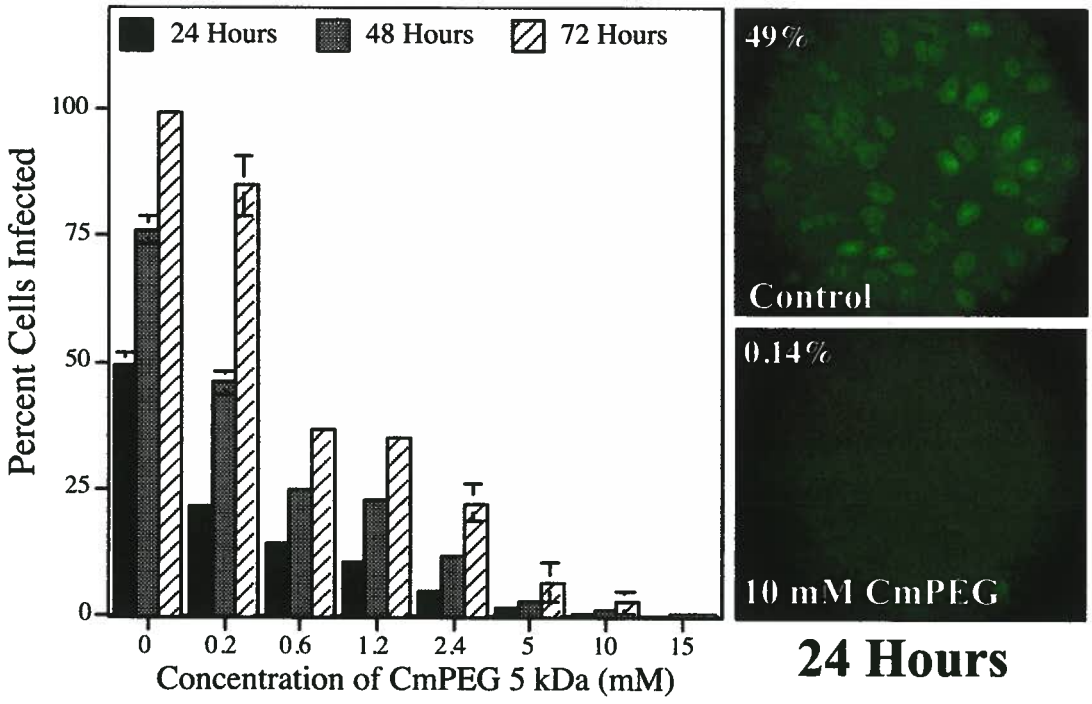


Figure 1.14. Grafting of CmPEG Directly to SV40 Effectively Inhibits Initial Viral Invasion and Infection. Subsequent to the initial decrease in invasion at 24 hours, propagation of the viral infection at 48 and 72 hours is also blunted. Shown on the right are photomicrographs showing SV40 T antigen staining in host cells. Increased T antigen staining indicates infection by SV40. The value shown is the percentage of T antigen positive cells exposed to control virus and mPEG-modified virus after 24 hours of culture. Figure modified from McCoy LL & Scott MD (116).

1.2.7.2 mPEG-Modification of Virus Host Cells

As an anti-viral prophylactic strategy, mPEG modification of virus host cells has recently been explored (116). Using indirect immunofluorescence to assess the number of cells expressing viral antigens, mPEG grafting to CV-1 cells (host cells for SV40) was shown to inhibit viral invasion in a dose-dependent manner. Similar to direct mPEG modification of the SV40 virion, this reduction was enduring. For example, when cells were challenged with a virus dose that produced infection in 50% of the cells at 24 hours, a 5 mM mPEG grafting concentration reduced the level of infection to 10%. Furthermore, at 72 hours post-infection using the same mPEG grafting concentration, approximately 25% of the modified cells versus 100% of the unmodified cells were infected (116).

mPEG grafting to host cells has also been evaluated using plaque assays, with the host cells for viruses that ranged in size from 30-200 nm and that used both fusion and receptor-mediated endocytosis as the mode of entry (135)(see Figure 1.15). The specific viruses studied included cytomegalovirus (CMV), Theiler's murine encephalomyelitis virus (TMEV), rat coronavirus (RCV), and mouse adenovirus (MAV). Similar to findings with the host cells for SV40, grafting of benzotriazole carbonate mPEG (BTCmPEG) to these host cells resulted in a dose-dependent reduction in the number of plaques. Importantly, BTCmPEG grafting to host cells for MAV was also shown to prevent virus infection over a range of virus doses (116). These findings suggest that mPEG grafting to host cells has the potential to be a broad-spectrum anti-viral prophylactic therapy, and work conducted within this thesis aims to extend these findings.

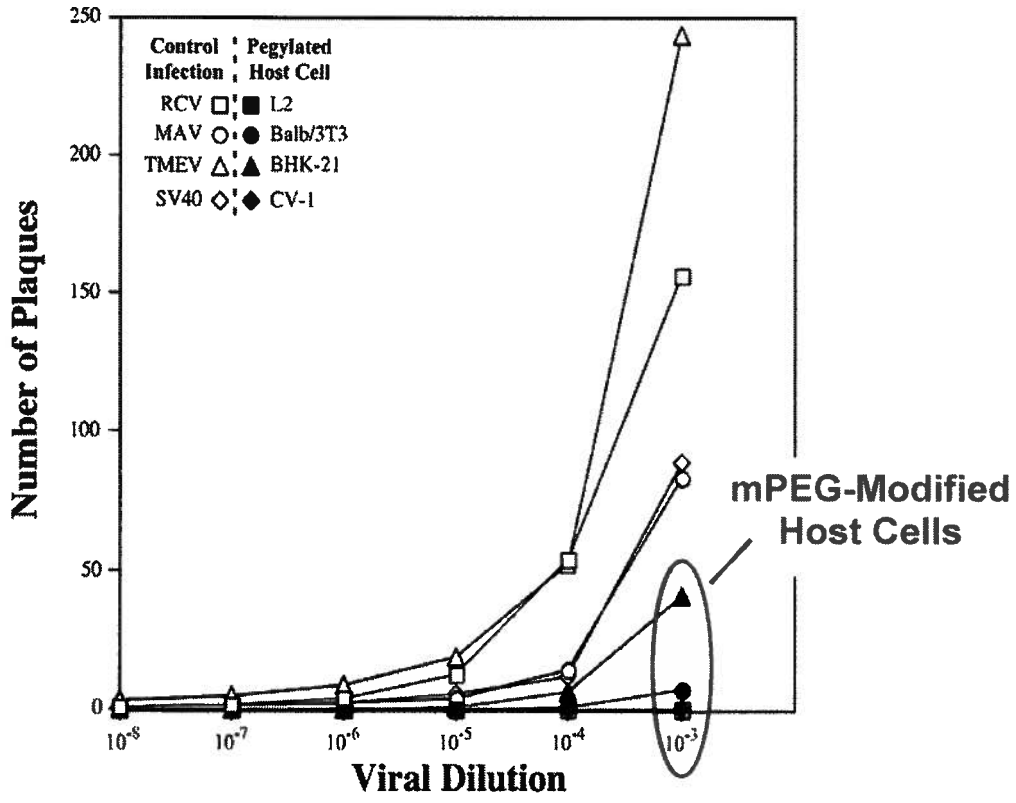


Figure 1.15. mPEG-Modification of Host Cells Prevents Infection by Several Viruses. mPEG modification of the host cell prevents against infection by rat coronavirus (RCV), mouse adenovirus (MAV), Theiler's murine encephalomyelitis virus (TMEV), and simian virus 40 (SV40). The number of plaques in mPEG-modified host cells is shown with enclosed symbols. Figure modified from McCoy LL & Scott MD (116).

Table 1.2. Comparison of Viruses Evaluated in the Context of Host Cell Modification including RSV. Note: The 2 and 20 kDa mPEG polymers extend approximately 5 and 50 nm, respectively.

Virus and *Family*	Virus Structure	Genome Type	Virion Size	Mode of Entry	Receptor	Distance Receptor Extends from the Cell Surface
Simian Virus 40 (SV40) *Papovaviridae*	Naked Icosahedral Capsid	double stranded circular DNA	45-55 nm	Receptor Mediated Endocytosis	Major Histocompatibility Molecule-1 (MHC-1)	7 nm
Mouse Adenovirus (MAV) *Adenoviridae*	Naked Icosahedral Capsid	double stranded linear DNA	70-90 nm	Receptor Mediated Endocytosis	murine homologue of Coxsackie and Adenovirus Receptor (mCAR)	4.6 nm
Rat Coronavirus (RCV) *Coronaviridae*	Enveloped Helical Capsid	positive single stranded RNA	80-160 nm	Fusion	Not yet identified but other family members use Aminopeptidase N (APN)	13.5 nm (APN)
Cytomegalovirus (CMV) *Herpesviridae*	Enveloped Icosahedral Capsid	double stranded linear DNA	150-200 nm	Fusion	Epidermal Growth Factor Receptor (EGFR)	3.7 nm
Theiler's Murine Encephalo-myelitis Virus (TMEV) *Picornaviridae*	Naked Icosahedral Capsid	positive single stranded RNA	28-30 nm	Receptor Mediated Endocytosis	Not yet identified but other family members use Intracellular Adhesion Molecule-1 (ICAM-1)	18.7 nm (ICAM-1)
Respiratory Syncytial Virus (RSV) *Paramyxoviridae*	Enveloped Helical Capsid	negative single stranded RNA	150-300 nm	Fusion	Not yet identified	Unknown

Families, structure, genomic material, size and mode of entry reference from (172). Receptor size references are as follows: MHC-1 (173), mCAR (174), APN (175), EGFR (176), and ICAM-1 (177).

CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS

2.0 EXPERIMENTAL HYPOTHESIS

Given that mPEG-modification of SV40 or the respective host cell surfaces for several viruses has been shown to prevent viral invasion, **as shown in Figure 2.1 (below), we hypothesized that normal RSV infection (A) could be prevented or attenuated by (B) covalent grafting of mPEG directly to the RSV virion, or (C) covalent grafting of mPEG to the host cell surface.**

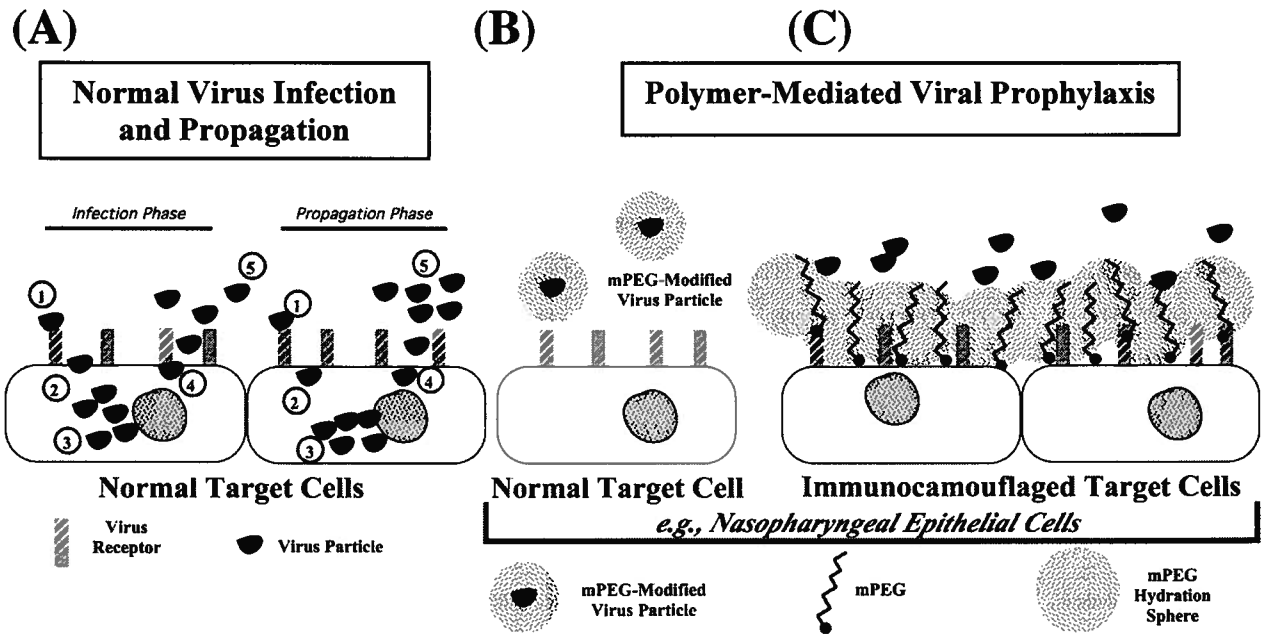


Figure 2.1. Schematic of Hypothesis. Viral Pathogenesis **(A)**: Virus recognizes and binds to cell receptor (1), fuses with the cell membrane and is internalized (2), undergoes multiple rounds of replication (3), progeny virus buds from the cell surface (4) into the extracellular environment (5) whereupon it infects new host cells and the cycle is repeated. Covalent mPEG grafting to the **(B) virus** or its **(C) host cells** prevents virus interaction with its surface receptor and the resulting infection. Figure modified from McCoy LL & Scott MD (116).

2.1 SPECIFIC AIMS

Within the context of the experimental hypotheses, we sought to evaluate the relationship between polymer linker chemistry, polymer length, and grafting density. In addition, we also sought to examine if mPEG grafting to the virus or host cell had any detrimental effects. These relationships were evaluated through the following specific aims:

1) To determine if direct virus modification or host cell modification prevents infection in non-polarized cells;
2) To determine the optimal linker chemistry, polymer length, and grafting density for virus particle modification that inhibits RSV infection and proliferation;
3) To determine the optimal linker chemistry, polymer length, and grafting density for host cell surface modification that inhibits RSV infection and proliferation;
4) To determine if direct viral modification or host cell modification prevents infection in polarized cells;
5) To determine the effect of mPEG-modification on the integrity of the RSV virion;
6) To demonstrate that mPEG grafts to the host cell surface;
7) To determine the effect of mPEG grafting to the host cell surface on cell viability.

2.2 STRATEGY

2.2.1 Virus Modification

To optimize linker chemistry, polymer length, and grafting density for viral modification and inactivation, two experimental approaches were employed. Initial experiments were performed using plaque assays with Long Strain A RSV, while subsequent experiments utilized green fluorescent protein expressing-RSV (GFP-RSV) and flow cytometry. GFP-RSV is a reverse engineered virus that expresses green fluorescent protein (GFP) early during infection.

This allows viral replication and proliferation to be monitored from 12 hours onwards, and permits the use of higher virus doses. Infected cells expressing GFP can be quantified by flow cytometry or fluorescent light microscopy. This is in contrast to clinical isolates that require several days of culture, and must be inoculated at lower concentrations to produce distinct syncytia and cytopathology.

For plaque assays, a standard virus concentration was modified over a range of mPEG grafting concentrations using different linker chemistries and polymer lengths. Polymer modified virus was then used to challenge non-polarized cells and syncytia were counted 5 days post-challenge. In subsequent experiments, to evaluate the efficacy of mPEG-modification over log scale increases in virus dose, SVA linker chemistry was used with both short and long chain polymers to modify increasing concentrations of GFP-RSV. Non-polarized cells were then challenged with mPEG-modified virus and the cells were harvested at time points between 12 and 96 hours post-infection. Subsequently, the cells were fixed and subject to flow cytometry to assess the level of infection and viral proliferation.

2.2.2 Host Cell Modification

Given the high efficacy of the SVA linker in viral modification, for host cell modification the effect of polymer length was examined with this chemistry. Further, as GFP-RSV infection could be followed from 12 hours onwards, we progressed directly to experiments in which non-polarized cells were modified with mPEG and challenged with this virus. Host cells were modified with either 2 or 20 kDa SVAmPEG, and then challenged with log scale doses of GFP-RSV. At time points between 12 to 96 hours, cells were then harvested, fixed, and subjected to flow cytometry, to assess the level of infection.

2.2.3 Efficacy of PEGylation Strategies in Polarized Cells

Given that RSV infects polarized cells *in vivo* (4), we examined if mPEG-modification of the RSV virion or the host cell similarly prevented infection in polarized cells. As RSV replicates less efficiently and does not form syncytia in immortalized polarized cells, we assessed the degree of protection conferred by either mPEG modification of the virus or host cell, by visualization and quantification of GFP-RSV infected cells. For these experiments concentrated GFP-RSV was used to partially overcome the diminished susceptibility of these cells to the virus. For mPEG modification, the optimal linker chemistry, polymer length, and grafting concentration determined in non-polarized cells was used to modify either GFP-RSV or the host cells. For viral modification, the modified virus was used to challenge polarized Madin-Darby canine kidney (MDCK) cells, while for host cell modification, the same cell line was modified and challenged with unmodified GFP-RSV. For both virus and host cell modification, green fluorescent protein expressing cells were counted at 24 and 48 hours post-infection, and the counts were compared to the unmodified control cells exposed to unmodified virus.

2.2.4. PEGylated RSV Virion Integrity

To determine if direct mPEG-modification of RSV disrupts the structure of the virus, GFP-RSV was modified with mPEG, concentrated, and subject to transmission electron microscope visualization.

2.2.5 mPEG-Host Cell Surface Modification and Toxicity

To verify that mPEG is grafted to the host cell surface, 5 kDa fluorescein-labeled mPEG was used to modify host cells. Cells were then visualized under both white and fluorescent light and digital images were captured. To assess if mPEG-grafting to host cells had any associated toxicity, host cells were modified with 20 kDa mPEG. From 0-48 hours post-modification, cell

monolayers were trypsinized and subject to propidium iodide (PI) staining and flow cytometry, or were left undisturbed and stained with trypan blue. Initial experiments with PI staining suggested that mPEG-modification may have some toxicity at high grafting concentrations. As mPEG-modified cells were resistant to trypsin digest, and therefore, required excess pipetting to shear the cells from the plate this finding was not surprising. Thus, we subsequently chose to stain intact cell monolayers with trypan blue to determine viability. Both PI and trypan blue are exclusion dyes and assess viability indirectly. The premise of both techniques is that a live cell has an intact or undisturbed cell membrane and can prevent or exclude dyes from entering the cell. The limitation of both methods is that a disturbed membrane or membrane with altered function does not ensure cell death. Cell membranes can be stressed and recover without killing the cell. PI staining is more sensitive than trypan blue because it is measured via flow cytometry compared to microscopy. However, trypan blue staining can be performed on intact cell monolayers, therefore, avoiding any negative effects of disrupting the cells. As both methods were utilized, the results of each method are presented.

2.3 APPLICATIONS OF mPEG TECHNOLOGY FOR RSV PROPHYLAXIS

mPEG modification in the context of RSV prophylaxis has two potential applications. First, direct mPEG-modification of RSV may provide a novel vaccine strategy (178). As mPEG-modification has been shown to increase the circulation half-life of therapeutic proteins and xenogenic blood cells, mPEG grafting to RSV, while inhibiting infection, may similarly lead to prolonged circulation *in vivo*. Given that mPEG does not modify all viral surface proteins, mPEG-modified RSV would slowly degrade in the blood stream and unmodified viral antigens would be presented to the host immune system. In this fashion, administration of mPEG-

modified RSV could result in prolonged exposure to unmodified viral antigens and potentially induce protective immunity. Secondly, activated mPEG may be used as a prophylactic nasal spray to modify the nasal epithelium (179). By modifying the epithelium, RSV and other viruses would not be able to establish the initial upper respiratory tract infection, and disease would be prevented. As an mPEG nasal spray would be non-toxic, relatively easy to administer, more effective and substantially less expensive than current RSV prophylactic methods, this strategy would be a novel cost-effective prophylactic method suitable for all children and at-risk patients.

2.4 GOAL OF RSV AND HOST CELL PEGYLATION STRATEGIES

While the ideal goal of either virus or host cell modification is to completely abrogate infection, this is not likely to be achievable. Therefore, as illustrated in Figure 2.2, the goal of PEGylation strategies, especially in the context of a nasal spray, is to reduce the inoculation dose to below the infectious dose (~112-448 pfu)(63-64). While not completely preventing the risk of infection, this would dramatically decrease the probability of clinically significant disease by keeping the infection at a level the host's immune system could more easily manage.

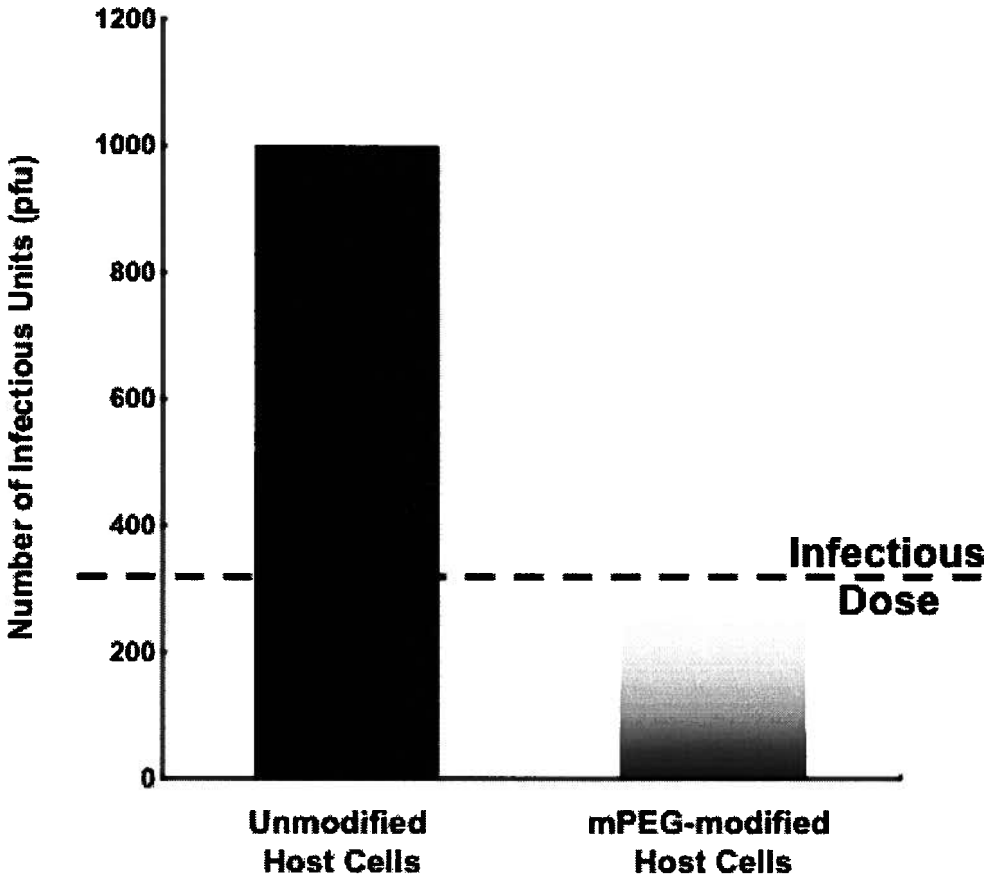


Figure 2.2. mPEG-Modification Strategies Aim to Decrease the Virus Dose To Below Disease Causing Levels. The goal of mPEG-modification of either the virus or host cells is not 100% protection, but rather reducing the inoculation dose to below the infectious dose.

CHAPTER 3: METHODS AND MATERIALS

3.0 OVERVIEW

Described in this chapter are the experimental methods used to test our hypotheses and specific aims. The experimental methods are presented according to the experimental strategy outlined in Chapter 2.

3.1 CELL AND VIRUS CULTURE

3.1.1 Cell Lines and Virus Strains

Vero, HeLa, Madin-Darby canine kidney (MDCK) cells, and RSV Long Strain A were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Recombinant green fluorescent protein (GFP) expressing RSV (GFP-RSV), clone 224 derived from the RSV A2 strain (81) was obtained from Dr. M. E. Peeples at the Children's Research Institute, University of Ohio.

Table 3.1. Comparison of Cell Lines

Comparison of Cell Lines				
Cell Line	**Origin**	**Cell Type**	**Morphology**	**Polarized/Non-Polarized**
Vero	African Green Monkey	Kidney	Epithelial	Non-polarized
HeLa	Human	Cervix	Epithelial	Non-polarized
MDCK	Cocker Spaniel	Kidney	Epithelial	Polarized

Table 3.2. Comparison of Virus Strains

Comparison of Virus Strains		
Virus Strain	**Origin**	**Length of Incubation Required for Detection in Culture**
RSV Long Strain A	Clinical Isolate	3-5 days
GFP-RSV Clone 224	Reverse Engineered from RSV Strain A2	12-20 hours

3.1.2 Cell Culture and Propagation

All cell lines were cultured in T-75 or T-175 tissue culture flasks (Becton Dickenson), Franklin Lakes, NJ, USA) and were grown in a humidified, 5% CO_2 incubator maintained at 37°C. For cell culture or propagation, Vero and MDCK cells were grown in minimal essential media (MEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, Sacramento, CA, USA), non-essential amino acids, MEM vitamin solution (1X), L-glutamine (2 mM), sodium pyruvate (1X), and gentamicin (0.05 mg/mL) (Invitrogen). HeLa cells were grown in high glucose (4.5 g/L D-glucose) Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% FBS (Gemini), GlutaMAX-I (2 mM), HEPES buffer (1 mM), penicillin (0.292 mg/mL), and streptomycin (100 μg/mL) (Invitrogen). Cells were maintained at a sub-culture ratio of 1:5 to 1:10 and were passaged every 3-5 days.

3.1.3 Preparation of Virus Stocks

For preparation of virus stocks, RSV Long Strain A and GFP-RSV were cultured in Vero and HeLa cells, respectively. To prepare virus stocks, cells were grown to 80-90% confluency in T-75 flasks (Becton Dickenson). Subsequently, the cells were rinsed twice with 10 mL of

phosphate buffered saline (PBS) (NaCl 137mM, KCl 2.68 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.76 mM) (Sigma-Aldrich, Oakville, ON, Canada) at pH 7.4, and then overlaid with RSV diluted in 5 mL of 2% FBS-supplemented media. Cells were infected at a multiplicity of infection (MOI), defined as the ratio of infectious virus particles to cells, of 0.1 (1 infectious unit per 10 cells). After the addition of virus containing media, cells were subject to standard infection conditions consisting of a 90 minute incubation at 37°C with gentle rocking every 15 minutes to ensure even distribution of the virus. Following the incubation period, the virus solutions were aspirated and the cells were overlaid with 15 mL of 2% FBS-supplemented media. Importantly, the tissue culture media for all GFP-RSV experiments did not contain phenol red, as this pH indicator can interfere with the fluorescent signal of GFP.

Virus cultures were examined daily under an inverted light microscope with a fluorescent lamp attachment (Olympus Microscope Model CK-40, Olympus America Incorporated, Center Valley, PA). For RSV Long Strain A, cultures were visualized under white light and were harvested once syncytia were abundant, usually 3-5 days post-infection. For GFP-RSV, infected HeLa cells were visualized under fluorescent light and were harvested once 90-100% of the cells expressed green fluorescent protein.

To harvest viral cultures, twenty to thirty, 3 mm diameter sterile glass beads (Corning Incorporated, Lowell, MA, USA) were added to the tissue culture flask. The cell monolayer was then disrupted by vortexing the flask for 1 minute, and the resulting virus suspension was transferred to a sterile 50 mL conical tube (Sarstedt, Montréal, QB, Canada). To remove cell debris from the virus lysate, the lysate was further subject to centrifugation at 1000 x *g* for 10 minutes at 4°C in an Allegra 6KR centrifuge (Beckman Coulter, Fullerton, CA, USA). The supernatant was then transferred to another 50 mL conical tube (Sarstedt), vortexed, and dispensed in 1 mL aliquots into 1.5 mL cryotubes (Nalgene Nunc International, Rochester, NY,

USA). The virus aliquots were subsequently flash frozen in a dry ice-ethanol bath and then stored at –80°C.

3.2 mPEG SPECIES

In this thesis, three mPEG linker chemistries were evaluated over polymer lengths from 2 to 20 kDa. These mPEG linker chemistries are illustrated in Figures 1.4-1.6, and include cyanuric chloride mPEG (CmPEG), succinimidyl carbonate mPEG (SCmPEG), and succinimidyl valerate mPEG (SVAmPEG). CmPEG (5 kDa) was purchased from Sigma-Aldrich (Sigma). SCmPEG (2 and 5 kDa), SVAmPEG (2, 5, and 20 kDa), and fluorescein-labeled SVAmPEG (5 kDa), were purchased from Laysan Bio Incorporated (Arab, AL, USA). All mPEG species were purchased as dry powders and were stored at -20°C until use.

3.3 mPEG-MODIFICATION OF RSV

For direct virus modification, RSV Long Strain A or GFP-RSV was diluted in 2% FBS-media. Subsequently, a standard volume of this diluted virus solution was dispensed into either 1.5 mL microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Canada) for plaque assay experiments for RSV or 15 mL conical tubes (Sarstedt) for GFP-RSV log scale virus dose experiments. The 2% FBS media was then adjusted to pH 7.8 and filter sterilized using a 20 mL syringe (Becton Dickenson) and 0.2 μm syringe filter (Pall Life Sciences, East Hills, NY, USA). This pH 7.8, 2% FBS-media was then added to each tube to adjust the volume. Volumes were adjusted such that the subsequent addition of increasing amounts of concentrated activated mPEG solution would yield final mPEG concentrations of 0, 2, 5, 7.5, 12.5, and 15 mM with a constant total volume and virus concentration (see Figures 3.1 and 3.2)

Concentrated activated mPEG solutions were prepared by adding 2% FBS-media at pH 7.8 to pre-weighed amounts of mPEG in sterile 15 or 50 mL conical tubes (Sarstedt). The mPEG-media mixture was then vortexed until all the mPEG dissolved, and working quickly, was dispensed into tubes containing virus designated for modification. Following the addition of activated mPEG to the virus solutions, solutions were vortexed and incubated at room temperature for 30 minutes. Subsequently, the mPEG-modified virus solutions were used to challenge unmodified host cells.

3.4 mPEG-MODIFICATION OF HOST CELLS

For host cell surface modification, cells were grown to 90-100% confluence and rinsed once with PBS. Subsequently, activated mPEG solutions with final concentrations of 0, 2, 5, 7.5, 12.5, and 15 mM were overlaid onto the cells (see Figure 3.3). Activated mPEG solutions were prepared by adding filter sterilized unsupplemented media, adjusted to pH 7.8, to pre-weighed amounts of mPEG in 15 or 50 mL conical tubes (Sarstedt). Cells were modified at constant volume to surface area ratio of 0.2 mL/cm^2 and were incubated with activated mPEG for 30 minutes at room temperature. Following modification, cells were rinsed with 2% FBS-media and then challenged with unmodified virus.

Importantly, for both virus and host cell PEGylation, the media contained proteins (virus-modification) and/or lysine residues (host cell-modification). Therefore, a proportion of the activated PEG will be consumed by the media. However, previous pilot studies comparing PEGylation of either the virus or host cell in media to PBS showed that the PBS overlay altered cell viability.

3.5 VIRAL PLAQUE ASSAYS

To evaluate the effect of different linker chemistries and/or polymer lengths in the context of virus modification, plaque assays were performed (see Figure 3.1). Vero cells were seeded at a density of 1.75×10^5 cells/well in 24-well tissue culture plates (Becton Dickenson). Cells were then incubated for 48 hours until 90-100% confluent. Prior to experimentation the cells were rinsed once with 0.5 mL of PBS. To assess the efficacy of mPEG-modification of RSV, approximately 250 plaque forming units (pfu) of RSV Long Strain A were modified per 1 mL of solution. Subsequently, for a given mPEG grafting concentration, 0.4 mL of mPEG-modified RSV solution was overlaid onto duplicate wells of the tissue culture plate. This resulted in a challenge dose of ~125 pfu per well or an MOI of ~0.0005. The cells were then incubated under standard infection conditions of 90 minutes at 37°C with gently rocking every 15 minutes. Next, the virus solutions were removed, and the cells were overlaid with a pre-warmed, 1:1 solution of sterile 1% methylcellulose (Sigma) in water to 2X MEM supplemented with 4% FBS, 2X vitamin solution, L-glutamine, sodium pyruvate, and gentamicin (0.10 mg/mL) (Invitrogen). Cells were then incubated for 5 days and syncytia were counted using an inverted light microscope (Olympus).

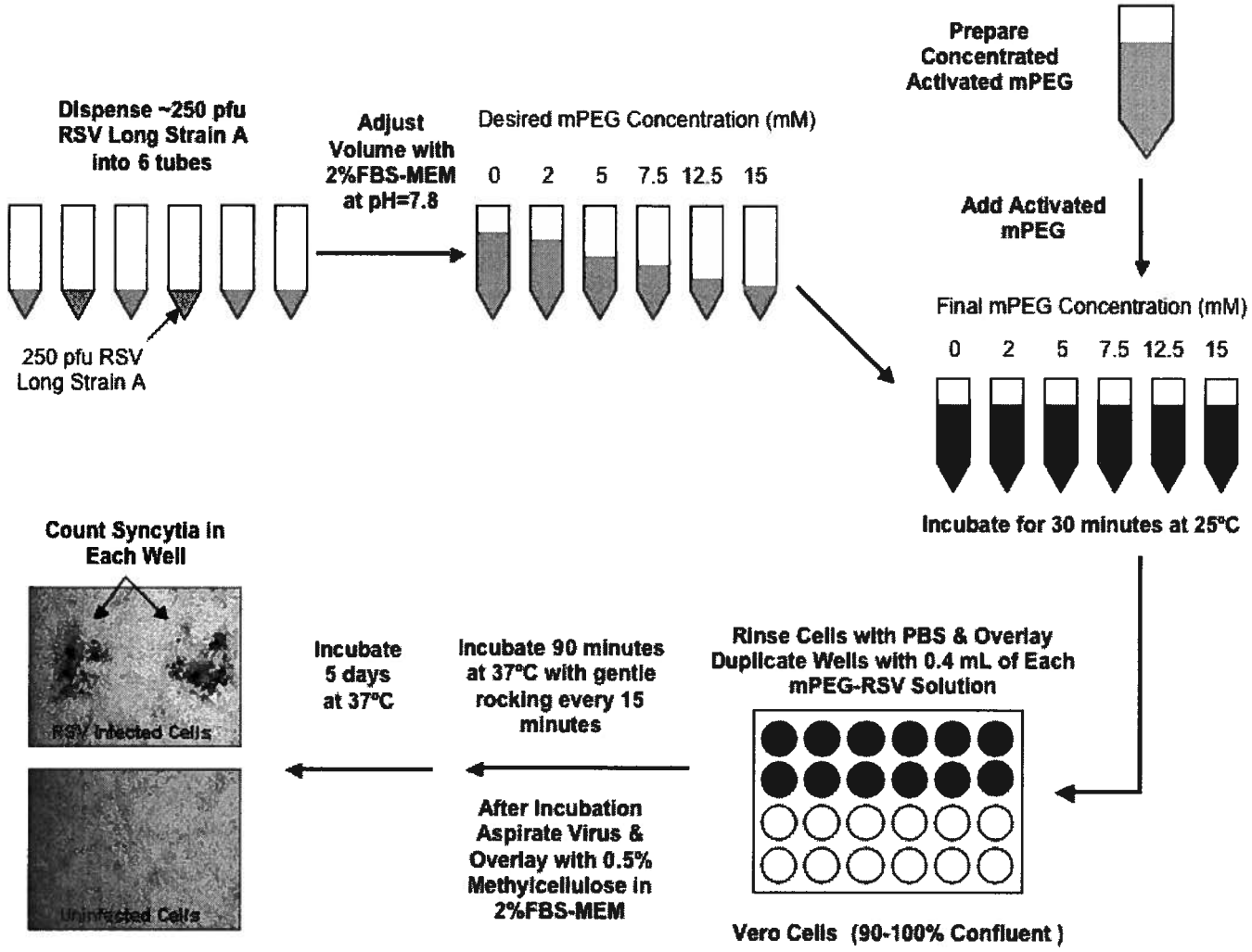


Figure 3.1. Schematic of mPEG-Modification of RSV Plaque Assay Procedure. See Section 3.5 for more details. Note: A negative control was also prepared and used to challenge cells in parallel. See Section 3.5 for more details.

3.6 GFP-RSV GROWTH CURVES

To determine appropriate time points for subsequent GFP-RSV experiments, growth curves were generated by infecting HeLa cells and quantifying the percentage of infected cells over time. For these experiments, cells were seeded in 96-well plates (Becton Dickenson) at a density of 4×10^4 cells per well and were incubated overnight until 90-100% confluent. Virus doses evaluated consisted of MOI = 0.5, 0.1, 0.05, 0.01, and 0, (50 pfu/100 cells, 10 pfu/100 cells, 5 pfu/100 cells, 1 pfu/100 cells, and 0 pfu/100 cells, respectively) based on 7.4×10^4 cells per well. Thus for a MOI = 0.5, RSV stocks were diluted to yield 3.7×10^4 pfu of GFP-RSV in an infection volume of 100 μL (0.1 mL) or a final concentration of 3.7×10^5 pfu/mL. Similarly, MOI = 0.1, 0.05, and 0.01 corresponded to 7.4×10^3 pfu, 3.7×10^3 pfu, and 7.4×10^2 pfu per 100 μL of media.

Prior to infection, cells were rinsed once with 100 μL of pre-warmed PBS and then overlaid with 100 μL of virus solution. Virus challenged plates were then incubated under standard infection conditions, and subsequently, the virus solutions were aspirated and replaced with 100 μL of 2% FBS-DMEM. At time points of 0, 2, 4, 6, 8, 10, 12, 18, 24, and 48 hours post-infection, cells were harvested and analyzed by flow cytometry as described by Hallak *et al.*, (81). Cells were harvested by aspirating the media and adding 45 μL of 0.25% trypsin-EDTA solution (Invitrogen). Cells were incubated at 37°C with gentle rocking every 5 minutes until the cells detached from the plate, usually 10-15 minutes. Subsequently, 80 μL of 10% FBS-DMEM was added to each well, and the cells were resuspended 10 times to create a single cell solution. Cells from a given well were then transferred to individual 1.5 mL microcentrifuge tubes (Fisher Scientific) and spun in a bench top microcentrifuge (Eppendorf, Westbury, NY, USA) at 300 x *g* for 5 minutes at room temperature.

The cell supernatant was removed and the cell pellet was re-suspended in 167 μL of PBS.

Subsequently, 333 μL of 2% paraformaldehyde (Fisher Scientific) in PBS at pH 7.4 was then added to each tube and the tubes were vortexed to ensure proper fixation of the cells. Cells were then transferred to 12 x 75 mm polystyrene flow cytometry tubes (Becton Dickenson) and subject to flow cytometry for GFP expression using a FACSCalibur Flow Cytometer (Becton Dickenson). Five thousand events were acquired on the high flow rate setting and analyses were conducted by gaiting on sham-infected cells (MOI = 0).

3.7 LOG SCALE VIRUS DOSE STUDIES

To further evaluate the efficacy of either virus or host cell modification, experiments were conducted with log scale increases in virus dose. To further assess the effect of polymer length, 2 and 20 kDa SVAmPEG was used to modify the virus or the host cells. For these studies, HeLa cells were prepared as described in the growth curve experiments and virus doses similarly consisted of MOI's = 0.5, 0.1, 0.05, 0.01 and 0. For all log scale studies, GFP-RSV was used.

For virus modification, GFP-RSV was modified with activated mPEG and 100 μL of mPEG-virus solution was used to challenge individual wells that had been pre-rinsed with PBS (see Figure 3.2). For host cell modification, 75 μL of activated mPEG was overlaid per well maintaining a constant volume to surface area ratio between plaque assays and log scale experiments (see Figure 3.3). Following mPEG grafting, cells were rinsed with 100 μL per well of 2% FBS-DMEM and then challenged with 100 μL per well of unmodified virus.

Virus challenged plates were incubated under standard infection conditions, and subsequently, the virus solutions were aspirated and replaced with 100 μL of 2% FBS-DMEM. At time points of 12, 24, 48, 72, and 96 hours post-infection, cells were harvested and analyzed by flow cytometry as described above (see Section 3.6). Importantly, flow cytometry

analyses were conducted by gaiting on cells exposed to mPEG-modified 2% FBS-DMEM (MOI = 0) for mPEG-modified virus, or for host cell modification, cells modified with mPEG and then sham infected (MOI = 0).

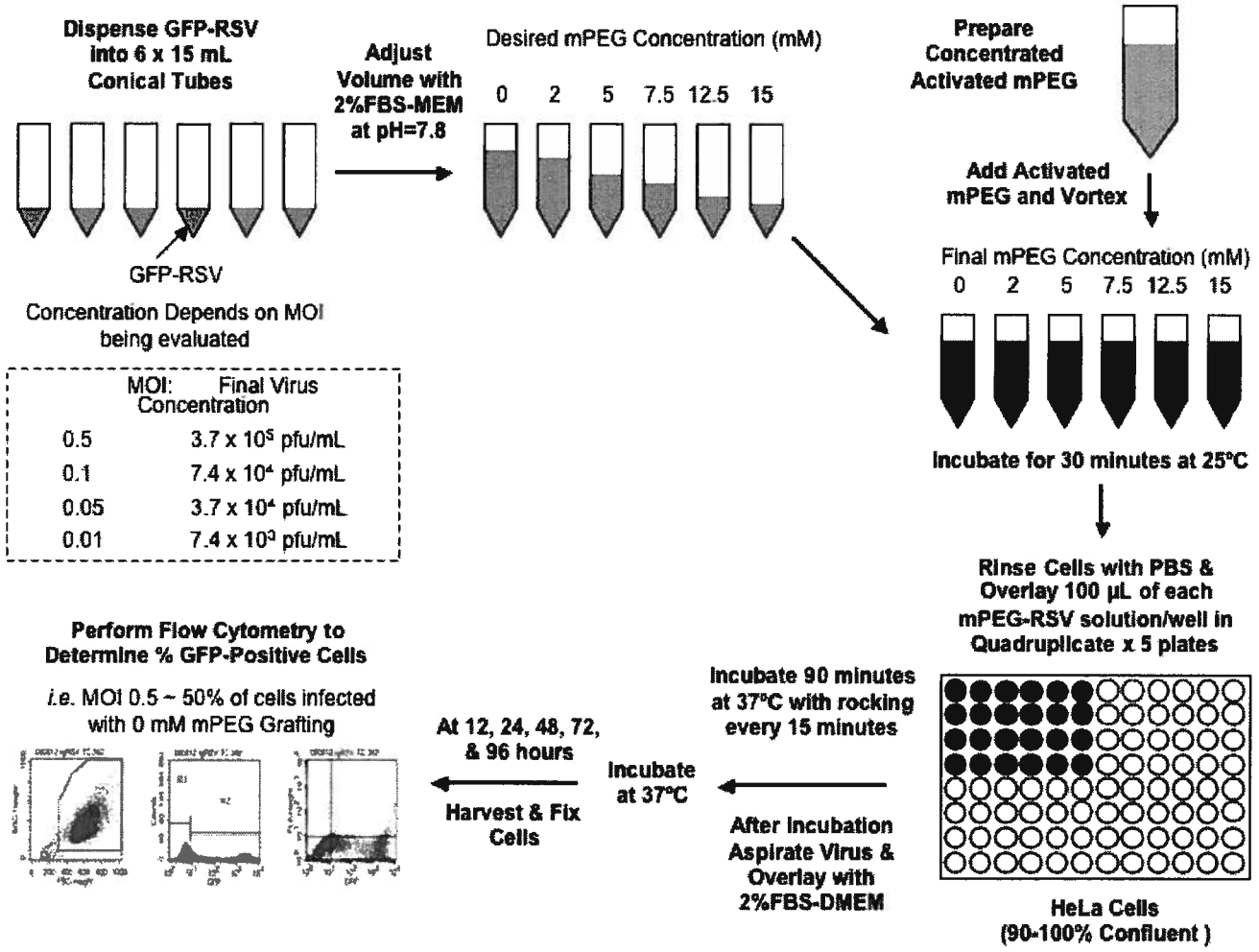


Figure 3.2. Schematic of mPEG-Modification of GFP-RSV Over Log Scale Virus Doses. Note: In this diagram, only one virus dose is shown. As well, for each time point, a negative control consisting of cells challenged with 2%FBS-media was prepared in parallel. This procedure was repeated for MOI = 0.1, 0.05, 0.01, and 0. For more details, see Section 3.7.

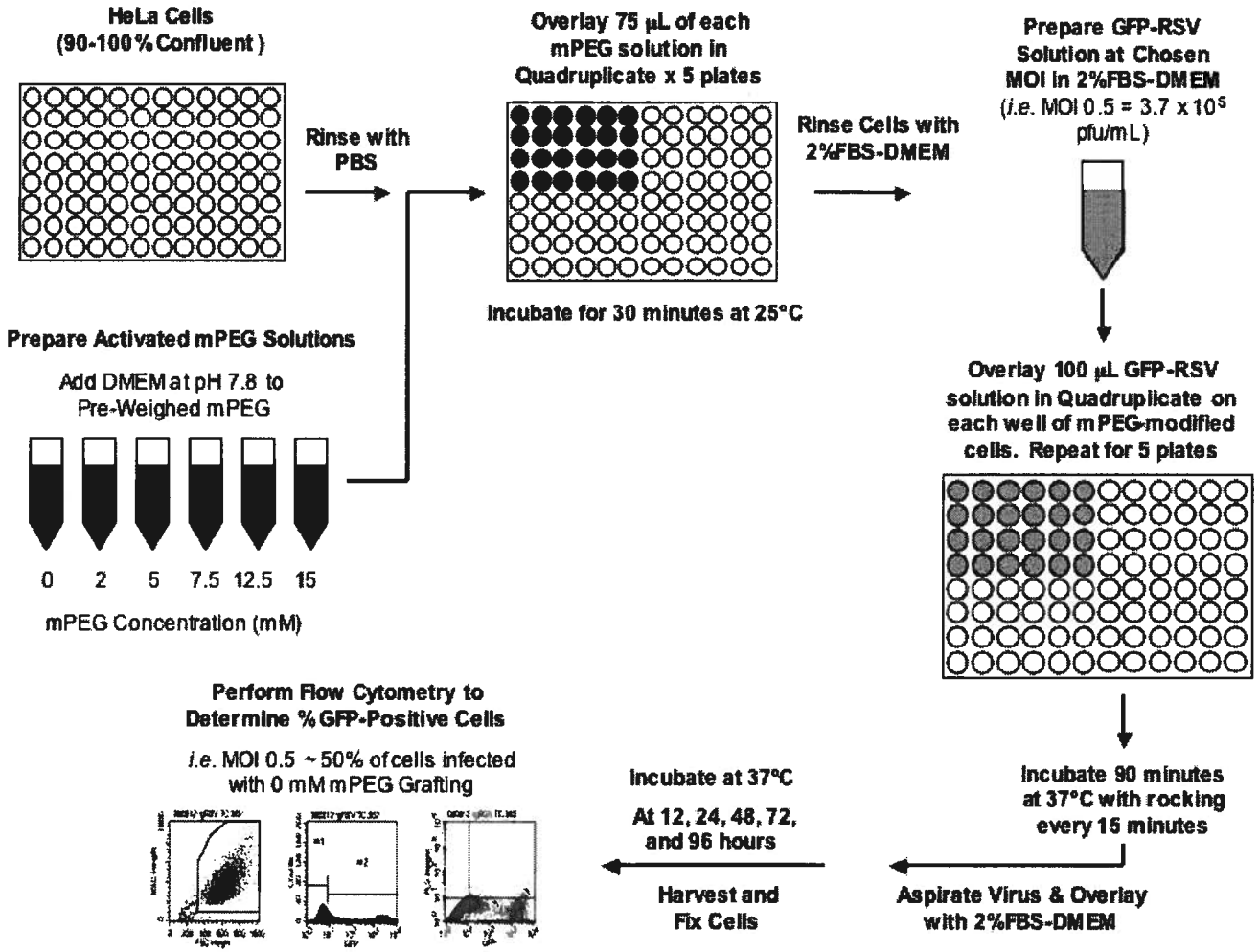


Figure 3.3. Schematic of mPEG-Modification of HeLa Cells Challenged with Log Scale Virus Doses. Note: In this diagram, only one virus dose is shown. As well, for time point, a negative control consisting of cells challenged with 2%FBS-media was prepared in parallel. This procedure was repeated for MOI = 0.1, 0.05, 0.01, and 0. For more details, see Section 3.7.

3.8 GFP-RSV INFECTION OF POLARIZED CELLS

To evaluate if mPEG modification of RSV or the host cells prevents infection in polarized cells, experiments were conducted using GFP-RSV and MDCK cells. MDCK cells were seeded in 96-well plates (Becton Dickenson) at a density of 4×10^4 cells per well and were incubated overnight until 90-100% confluent.

To overcome the decreased susceptibility of this cell line to RSV (77), a high virus concentration consisting of 6.15×10^5 pfu/well or MOI = 8 was used for both virus and host cell modification experiments. Based on the results of the log scale virus dose studies, a standard mPEG grafting concentration of 7.5 mM was employed for both virus and host cell modification, and GFP-RSV or MDCK cells were modified under standard conditions. For virus modification, GFP-RSV was pegylated with 2 kDa SVAmPEG, while for host cell modification, MDCK cells were pegylated with 20 kDa SVAmPEG.

Prior to mPEG-virus challenge or host cell modification, cells were rinsed once with 100 μL of PBS. For virus modification, 100 μL of mPEG-virus solution was overlaid on the cells. For host cell modification, cells were rinsed with 100 μL of 2% FBS-MEM media following cell derivitization and then challenged with 100 μL of concentrated unmodified GFP-RSV. Cells were incubated with the virus solutions for 12 hours at 37°C, and subsequently, the virus solutions were removed and the cells were overlaid with 100 μL of 2% FBS-MEM. Cells were then incubated for an additional 48 hours, and at both 24 and 48 hours post-infection, GFP expressing cells were visualized and counted using an inverted light microscope with fluorescent lamp attachment (Olympus).

3.9 TRANSMISSION ELECTRON MICROSCOPY

To ensure that mPEG-modification of RSV did not disrupt the virion integrity or result in aggregation of virus particles, transmission electron microscopy (TEM) using a Hitachi H7600 (Hitachi High Technologies America Inc., Pleasanton, CA, USA) microscope was performed on mPEG-modified GFP-RSV.

A total of 2.775×10^6 pfu of GFP-RSV was modified at mPEG concentrations of 0, 2, 7.5, and 15 mM. GFP-RSV was modified with 2 kDa SVAmPEG in 7.5 mL of 2% FBS-media at pH 7.8. This resulted in a virus concentration equivalent to an MOI = 0.5 for the log scale virus dose studies. After modification, the virus solutions were loaded into Amicon Ultra-15 centrifugal filter units with a molecular weight cut-off of 100 kDa (Millipore Corporation, Bedford, MA, USA). The filter units were subsequently spun in an Allegra 6KR centrifuge (Becton Dickenson) at 3500 x *g* for 20 minutes at room temperature. The resulting virus concentrate had an approximate volume of 200 μL, and was transferred to a sterile 0.5 mL microcentrifuge tube (Fisher) and placed on ice.

For sample preparation, 10 μL of the mPEG-virus concentrate was then diluted in 90 μL of 0.01% bovine serum albumin (Sigma) in water. From this stock, 5 μL of the sample was placed on a Formvar carbon coated copper grid (400 mesh) (Cedarlane Laboratories Ltd., Burlington, ON, Canada) and allowed to adhere for 90 seconds. Subsequently, 10 μL of 2% phosphotungstic acid (PTA) (Fisher Scientific) was added on top of the virus solution, and the virus and PTA mixture was allowed to incubate for 60 seconds. The virus-PTA mixture was then blotted off the grid with filter paper (Fisher), and the grids were stored at room temperature until TEM visualization. Individual virus particles were then visualized and images were captured at 100, 000 x magnification.

3.10 FLUORESCEIN-mPEG MODIFICATION OF CELLS

To verify that mPEG was grafting to the host cell surface, HeLa cells were cultured in 96-well plates (see Section 3.5) and modified with a mixture of 5% fluorescein-labelled and 95% unlabelled 5 kDa SVAmPEG (Laysan Bio). Cells were modified under standard grafting conditions in unsupplemented DMEM media adjusted to pH 7.8 and lacking phenol red. Grafting concentrations consisted of 0, 2, 7.5, and 15 mM. As a control, the activated fluorescein-mPEG and unlabelled mPEG mixture was left to hydrolyze for 4 hours at room temperature and similarly used to modify cells. Following mPEG-modification, the cells were rinsed three times with 2% FBS-DMEM (without phenol red) and visualized under white and fluorescent light using an inverted light microscope (Olympus). Digital images were captured using a QICAM FAST camera (Q-Imaging Corporation, Surrey, BC, Canada) mounted on an inverted light microscope with OpenLab version 5.5.0 software (Improvision, Perkin Elmer, Coventry, England).

3.11 CELL VIABILITY OF mPEG-MODIFIED CELLS

To assess cell viability, propidium iodide (PI) and trypan blue staining was performed. HeLa cells were seeded in 48-well plates (Becton Dickenson) at a density of 8×10^4 cells per well, and incubated overnight until 90-100% confluent. Cells were then rinsed once with 200 μL of PBS and modified with 150 μL of activated 20 kDa SVAmPEG. Grafting concentrations included 0, 2, 7.5, and 15 mM, and 20 kDa SVAmPEG was chosen as it provided enhanced cell protection relative to other mPEG species. Following mPEG-modification, cells were rinsed and overlaid with 200 μL of 2% FBS-DMEM and returned to the incubator.

At 0 and 24 hours post-modification, the media was aspirated from the cells and 90 μL of trypsin (Invitrogen) was added to each well. Cells were then incubated at 37°C for 10 minutes.

Subsequently, 160 μL of 10%FBS-DMEM was added to each well, and the cells were pipetted 10-15 times to dislodge the cells and make a single cell solution. The cells were then transferred to a sterile 1.5 mL microcentrifuge tube (Fisher Scientific) and centrifuged at room temperature for 5 minutes at 300 x *g*. The supernatant was aspirated and the cells were rinsed twice with 200 μL of cold PBS, followed by re-suspension, and centrifugation at room temperature for 5 minutes at 300 x *g*. After the second rinse, the cells were resuspended in 100 μL of cold PBS and 2 μL of 50 μg/mL propidium iodide was added to each tube. The cells were then incubated in the dark for 15 minutes, and added to flow tubes containing 400 μL of PBS. After mixing the cells in the flow tubes, the cells were subject to flow cytometry analysis on a FACSCalibur Flow Cytometer (Becton Dickenson). Five thousand events were acquired on the high flow rate setting and analyses were conducted by gaiting on unmodified cells.

To avoid toxicity associated with disrupting the cell monolayer, HeLa cells were modified with mPEG and subject to in-plate trypan blue staining. In this procedure, intact cell monolayers were stained as mPEG-modified cells were resistant to trypsin digest. Therefore, to dislodge the cells from the plate, mPEG-modified cells required extra pippetting and manipulation. As PI staining results suggested that this could potentially result in increased toxicity and/or a decrease in total cell number, in-plate staining avoided these effects. Thus, HeLa cells were seed and modified as described for PI staining.

At 0, 24, and 48 hours post-modification, the media was aspirated from the cells and 100 μL of 0.2% trypan blue (Sigma) in DMEM was added to each well. The plates were then incubated at 25°C for 5 minutes, and rinsed gently with 200 μL of 2% FBS-DMEM. Next, the cells were overlaid with 200 μL of 2% FBS-DMEM and blue stained cells were counted under an inverted microscope (Olympus).

To obtain an estimate of the percent viability, after trypan blue staining, the unmodified (0 mM) control cells were subject to trypsin digest and counted. This was performed by removing the media from each well and adding 90 μL of 0.25% trypsin-EDTA (Invitrogen). The plates were then incubated at 37°C until the cells detached. Next, 160 μL of 10% FBS-DMEM was added to each well and the cells were re-suspended 10 times to make single cell solution. Cells from each well were then pooled in a sterile 2 mL microcentrifuge tube (Fisher), and 10 μL of this solution was added to a hemacytometer (Hausser Scientific, Horsham, PA, USA). Cells were then counted and the total number of cells per well was calculated. Subsequently, the number of blue-stained dead cells was subtracted from the total number of cells as estimated from the total number of unmodified cells, and percent viability was calculated. Results were then expressed as both the total number of dead cells per well, and as estimated percent viability.

3.12 STATISTICAL ANALYSIS

For plaque assay experiments, a minimum of three replicates were performed and, within a given replicate, each condition was evaluated in duplicate. For the log scale virus dose studies, GFP-RSV infection of polarized cells, and trypan blue cell viability experiments, two replicates were performed, and each condition was evaluated in quadruplicate. For all analyses, SPSS v12 statistical software (Statistical Products and Services Solutions, Chicago, IL, USA) was used with a two-tailed p-value < 0.05 considered statistically significant.

Overall mean values and standard error for each experimental condition were calculated by combining all replicates. For statistical analyses, data from the log scale GFP-RSV experiments was normalized to the unmodified (0 mM) control and compared. For all other experimental procedures, data did not require any transformation, and raw data were used in the analysis.

Subsequently, for comparison of three or more means, a one-way analysis of variance (ANOVA) was performed. When significant differences were found, a post-hoc Tukey test was used for pair-wise comparison of means. When only two means were compared, as in the log scale studies at a given mPEG concentration, student-t tests were performed.

CHAPTER 4: RESULTS: mPEG-MODIFICATION OF RSV

4.0 OVERVIEW

The results are divided into two chapters: Chapter 4, mPEG-modification of RSV, and Chapter 5, mPEG-modification of the host cells. Of note, experiments characterizing GFP-RSV are included in the mPEG-modification of RSV chapter.

Based on previous studies with mPEG-modified SV40, we hypothesized that RSV infection could be prevented or attenuated by covalent grafting of mPEG directly to the RSV virion (see Figure 4.1). As detailed below, we show that mPEG-modification of RSV is a highly effective strategy for inhibiting infection. While this approach may not be directly applicable for respiratory viruses, it could be of value in areas such as blood banking (*i.e.* CMV inactivation) and/or vaccine development (*i.e.* RSV vaccine).

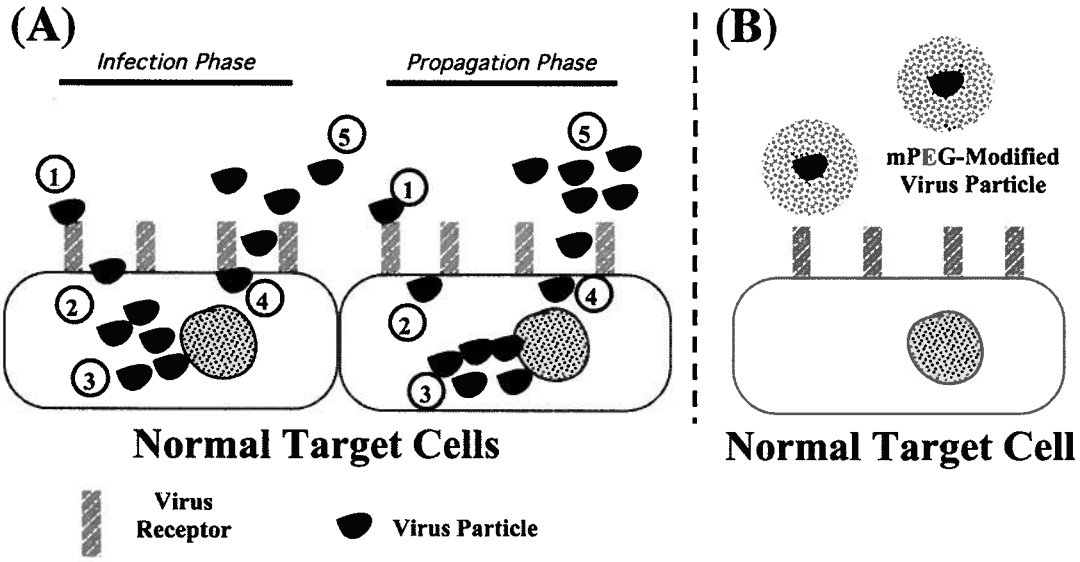


Figure 4.1. Schematic of mPEG-Modified RSV Hypothesis. Viral pathogenesis **(A)**: RSV recognizes and binds to the cell surface receptor (1), fuses with the cell membrane and is internalized (2), undergoes multiple rounds of replication (3), progeny virus buds from the cell surface (4) into the extracellular environment (5) whereupon it infects new host cells and the cycle is repeated. **(B)** Covalent grafting of mPEG directly to RSV prevents its interaction with its surface receptor and the resulting infection.

4.1 mPEG-MODIFICATION OF RSV PLAQUE ASSAY STUDIES

4.1.1 mPEG-Modification of RSV Prevents Infection

To determine if mPEG-modification of RSV inhibited viral infection, we initially performed plaque assays with 5 kDa CmPEG. In these experiments, Long Strain A RSV was modified with mPEG and then used to challenge non-polarized Vero cells. Following viral challenge, cells were incubated for 5 days, and the number of syncytia or plaques were counted. In accordance with previous studies by McCoy *et al.* (2005) (116), soluble mPEG had minimal effect on the level of infection; however, as shown in Table 4.1 and Figure 4.2, CmPEG modification of RSV with grafting concentrations from 0 to 15 mM resulted in a dose-dependent decrease in the number of syncytia. At a 5 mM grafting concentration, the number of syncytia was modestly (~29%) though significantly reduced relative to the unmodified control (0 mM) ($p<0.015$). Furthermore, mPEG grafting at concentrations of 7.5, 12.5, and 15 mM resulted in further reductions by 45.6, 70.7, and 85.7 %, respectively, in the number of syncytia. Thus, mPEG-modification inhibits RSV infection in a dose-dependent manner.

Table 4.1. Modification of RSV with 5 kDa CmPEG Prevents Infection in a Dose Dependent Manner.

Effect of 5 kDa CmPEG Grafting to RSV on the Average Number of Syncytia Per Well		
mPEG Grafting Concentration	Average Number of Syncytia Per Well (Value ± Standard Error (SE))	Percent Reduction in the Number of Syncytia Per Well
0 mM	114 ± 8	0 %
2 mM	109 ± 5	4 %
5 mM	*81 ± 4	*29 %
7.5 mM	*62 ± 3	*46 %
12.5mM	† 33 ± 8	† 71 %
15 mM	† 16 ± 4	† 86 %

Values are averages of three separate experiments performed in duplicate.
* significantly different from the 0 mM control and 2, 12.5, and 15 mM ($p<0.015$).
† indicates significantly different from 0, 2, 5 and 7.5 mM grafting concentrations ($p<0.038$).

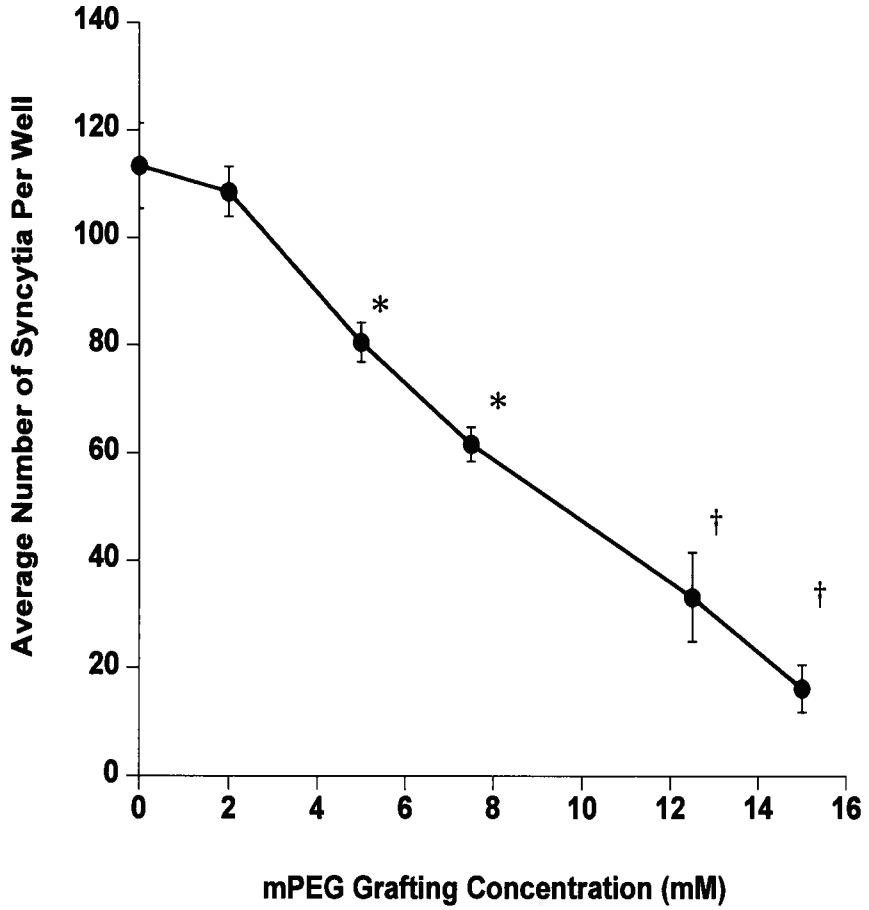


Figure 4.2. Modification of RSV with 5 kDa CmPEG Prevents RSV Infection in a Dose-Dependent Manner. RSV was modified with 5 kDa CmPEG and then used to challenge Vero cells in a plaque assay. At a grafting concentration of 5 mM, mPEG modification resulted in a significant decrease in the number of syncytia ($p<0.015$). Modification of RSV with higher concentrations of mPEG resulted in a further decrease in the number of syncytia, indicating a dose-dependent response.
Values shown are mean $\pm$ SE for three separate experiments performed in duplicate.
* significantly different from the 0 mM control and 2, 12.5, and 15 mM ($p<0.015$).
† indicates significantly different from 0, 2, 5 and 7.5 mM grafting concentrations ($p<0.038$).

4.1.2 Effect of mPEG Linker Chemistry and Grafting Density on RSV Infection Determined by Plaque Assay

Based on our initial results with CmPEG, we further investigated the effect of mPEG linker chemistry on inhibition of RSV infection by comparing several chemistries at standard polymer lengths of 2 or 5 kDa. As shown in Figure 4.3 and Table 4.2, modification of RSV with SC and SVA linker chemistries at the 2 kDa polymer length, resulted in a dose-dependent decrease in the number of syncytia. At a grafting concentration of 2 mM or greater, both SC and SVAmPEG significantly decreased the number of syncytia relative to the unmodified control (0 mM) ($p<0.001$). Importantly, at the grafting concentrations of 2, 5, and 7.5 mM, SCmPEG decreased infection by 30.3, 77.7, and 96.2 %, respectively, while SVAmPEG reduced the level of infection by 57.8, 99.1, and 100%. Comparing SC and SVAmPEG at these grafting concentrations, SVAmPEG significantly reduced the number of syncytia relative to SCmPEG ($p<0.007$), while at high, 12.5 and 15 mM mPEG concentrations, both mPEG species were able to completely prevent infection (100% reduction).

Examining linker chemistries at the 5 kDa polymer length, modification of RSV with CmPEG, SCmPEG, or SVAmPEG resulted in a dose-dependent decrease in the number of syncytia. CmPEG caused a significant reduction at grafting concentrations of 5 mM and higher ($p<0.02$), while both SC and SVAmPEG significantly reduced the number of syncytia at 2 mM and higher concentrations ($p<0.007$ and 0.001, respectively).

When comparing the efficacy of the different linker chemistries at the 5 kDa polymer length, it was found that SVAmPEG provided significantly greater inhibition of RSV at all grafting concentrations ($p<0.026$) with the exception of 15 mM. At this 15 mM concentration, all mPEG species gave near complete protection (see Figure 4.4). SVAmPEG decreased infection by 71.6% at the 2 mM concentration and completely abolished infection (100% reduction) at higher grafting concentrations. SCmPEG provided significantly greater protection

than CmPEG at the 5 mM grafting concentration (51.5% vs. 29% reduction, respectively)($p<0.001$), but at all other concentrations was not significantly different from CmPEG. Importantly, at moderate to high mPEG concentrations of 7.5-15 mM, grafting of either CmPEG or SCmPEG reduced infection by 45.6% and 63.1% to 85.7 and 88.9%, respectively, but did not completely prevent infection. In contrast, grafting of SVAmPEG, at these concentrations, completely abolished infection.

To understand this enhanced efficacy with SVAmPEG, it is important to consider the hydrolysis half-lives of the different mPEG species. As shown in Table 1.1, SVAmPEG has a hydrolysis half-life of 33.6 minutes compared to ~10 minutes and 20.4 minutes for CmPEG and SCmPEG, respectively. Thus, during the 30 minute mPEG grafting period, more SVAmPEG will remain active in solution and be able to graft to the virus. This will result in a more dense mPEG layer on the virion surface and, in turn, more significantly inhibit the virion from being able to infect its host cell. In summary, all mPEG linker chemistries at both the 2 and 5 kDa polymer lengths inhibited RSV infection. However, at both polymer lengths, the SVA linker chemistry provided enhanced protection.

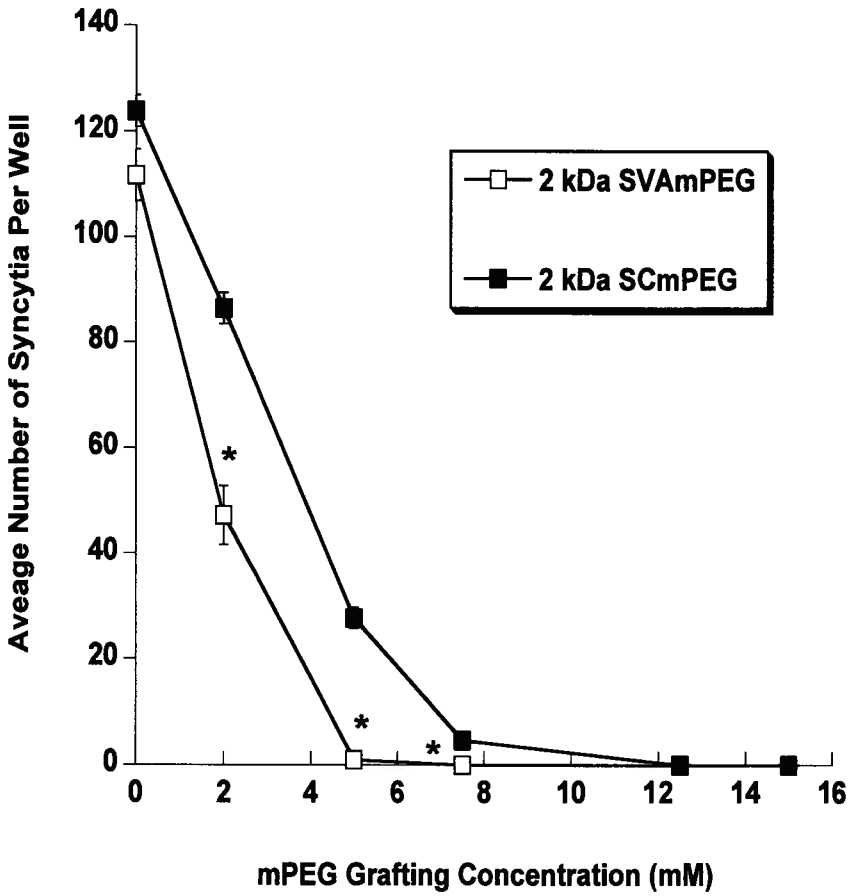


Figure 4.3. Modification of RSV with 2 kDa SVAmPEG Provided Enhanced Protection Compared to 2 kDa SCmPEG. RSV was modified with 2 kDa SVAmPEG or SCmPEG and then used to challenge Vero cells. Both 2 kDa mPEG species significantly decreased RSV infection in a dose-dependent manner ($p<0.001$), and SVAmPEG provided significantly better protection than SCmPEG ($p<0.007$).
Values shown are mean $\pm$ SE for three separate experiments performed in duplicate.
* indicates significantly different from SCmPEG ($p<0.007$).

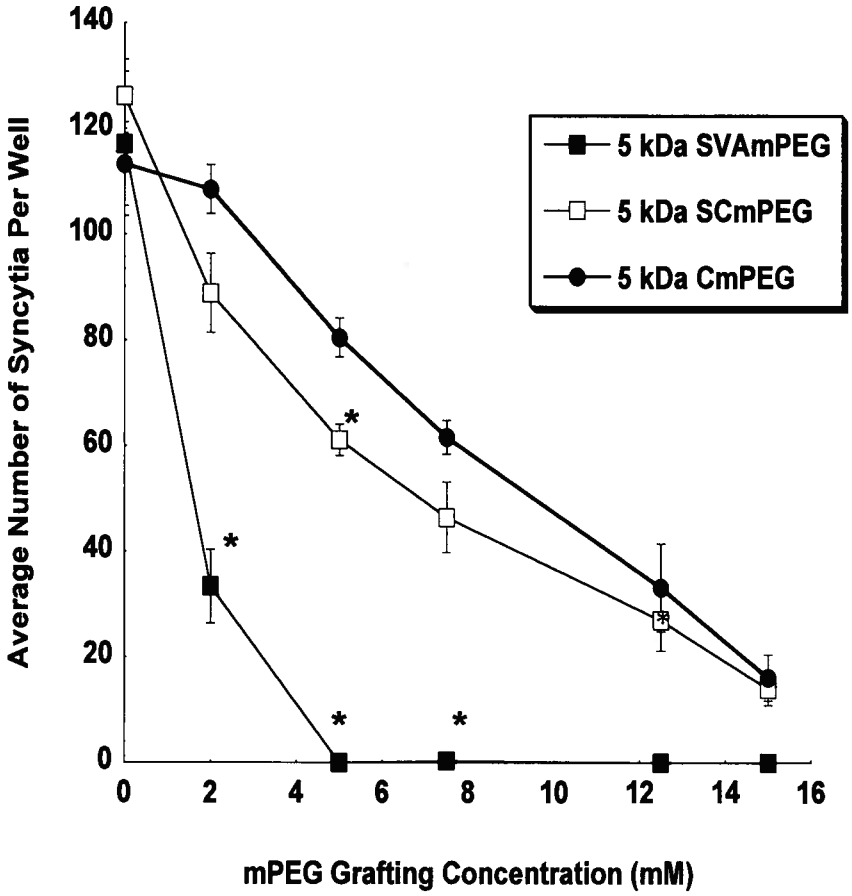


Figure 4.4. Modification of RSV with 5 kDa SVAmPEG Provided Enhanced Protection Relative to Other 5 kDa mPEG Species. RSV was modified with 5 kDa SVAmPEG, SCmPEG, or CmPEG, and then used to challenge Vero cells in a plaque assay. All 5 kDa mPEG species decreased RSV infection in a dose-dependent manner, however, SVAmPEG provided significantly greater inhibition of RSV infection relative to all other mPEG species ($p<0.026$). Values shown are mean $\pm$ SE for three separate experiments performed in duplicate.
* indicates significantly different from all other mPEG species ($p<0.026$).

Table 4.2. For mPEG-Modification of RSV, SVA Linker Chemistry Provides Enhanced Inhibition of Infection Relative to Other Linker Chemistries.

2 kDa Polymer Length: Effect of Linker Chemistry on the Average Number of Syncytia Per Well and the Percent Reduction						
	mPEG Grafting Concentration (mM)					
Linker Chemistry	0	2	5	7.5	12.5	15
SCmPEG	124 ±3 (0 %)	*86 ± 3 (30 %)	*28 ± 2 (78 %)	*5 ± 1 (96 %)	*0 ± 0 (100 %)	*0 ± 0 (100 %)
SVAmPEG	112 ± 5 (0 %)	†47 ± 6 (58 %)	†1 ± 1 (99 %)	†0 ± 0 (100 %)	*0 ± 0 (100 %)	*0 ± 0 (100 %)
5 kDa Polymer Length: Effect of Linker Chemistry on the Average Number of Syncytia Per Well and the Percent Reduction						
CmPEG	114 ± 8 (0 %)	109 ± 5 (4 %)	*81 ± 4 (29 %)	*62 ± 3 (46 %)	*33 ± 8 (71 %)	*16 ± 4 (86 %)
SCmPEG	126 ± 7 (0 %)	*89 ± 7 (30 %)	*†61 ± 3 (52 %)	*47 ± 7 (63 %)	*27 ± 6 (79 %)	*14 ± 3 (89 %)
SVAmPEG	117 ± 14 (0 %)	*†33 ± 7 (72 %)	*†0 ± 0 (100 %)	*†0.3 ± 0.3 (99 %)	*†0 ± 0 (100 %)	*0 ± 0 (100 %)

Values represent the number of syncytia per well at five days post-infection, and are expressed as mean ± standard error. Values represent averages of three experiments performed in duplicate. Values in brackets represent the percent reduction relative to the 0 mM control.

* significantly different from the 0 mM control ($p<0.02$).

† significantly different from all other linker chemistries at the same polymer length ($p<0.025$).

4.1.3 Effect of Polymer Length and Grafting Density Determined By Plaque Assay

To evaluate the effect of mPEG polymer length on RSV inactivation, we compared polymers ranging in length from 2 to 20 kDa using the same linker chemistry. As shown in Figure 4.5 and Table 4.3, we first compared 2 and 5 kDa SCmPEG. Grafting of either mPEG species resulted in a dose-dependent decrease in the number of syncytia. For both polymer lengths, this decrease was significantly different from the control (0 mM) at 2 mM or higher grafting concentrations ($p<0.002$), with 2 mM grafting resulting in an ~30% decrease in the number of syncytia for both polymer lengths. At grafting concentrations of 5, 7.5, and 12.5 mM, 2 kDa SCmPEG further decreased the number of syncytia by 77.7, 96.2, and 100%, respectively. At these same grafting concentrations, 5 kDa SCmPEG reduced the number of syncytia by 51.5, 63.1, and 78.6 %, respectively. Comparing the two polymer lengths at these concentrations, 2 kDa SCmPEG grafting caused a more significant reduction in the number of syncytia than 5 kDa SCmPEG ($p<0.012$); however, at the 15 mM grafting concentration, both mPEG species were not significantly different. Importantly, at both 12.5 and 15 mM grafting concentrations, 2 kDa SCmPEG completely inhibited infection (100% reduction), while 5 kDa SCmPEG did not (78.6% and 88.9% reduction for 12.5 and 15 mM, respectively). Thus, in the context of SCmPEG, the shorter 2 kDa species provides greater inhibition of RSV infection than the 5 kDa species.

Subsequently, we evaluated the effect of polymer length with the SVA linker chemistry (see Figure 4.6 and Table 4.3). In plaque assay experiments, we compared 2, 5, and 20 kDa SVAmPEG, and with all polymer lengths, mPEG grafting resulted in a dose-dependent decrease in the number of syncytia. This decrease was significantly different from the unmodified (0 mM) control at grafting concentrations of 2 mM or greater ($p<0.001$), with 2 mM grafting reducing the number of syncytia by more than 57% with all polymer lengths. At all mPEG grafting concentrations except 5 mM, all three polymer lengths provided similar levels of

protection. At the 5 mM grafting concentration, both the 2 and 5 kDa species provided enhanced protection with greater than 99% reduction, compared to the 20 kDa polymer which reduced infection by 82.9% reduction ($p<0.025$). Importantly, at high mPEG concentrations of 12.5 and 15 mM, all SVAmPEG species completely prevented RSV infection (100% reduction).

In summary, shorter mPEG polymers provided greater inhibition of RSV infection. This is particularly evident with the SC linker chemistry. With SCmPEG, the 2 kDa polymer provides significantly greater protection than the 5 kDa species ($p<0.012$), and is capable of completely abolishing infection. Differences were also found with SVAmPEG, at the 5 mM grafting concentration, as both the 2 and 5 kDa polymers provided enhanced protection (~99% reduction) relative to the 20 kDa polymer (83 % reduction). These differences are likely the result of larger polymers exerting more self-exclusion once bound to the virion surface. With longer polymers, this will result in decreased grafting density relative to the shorter mPEG polymers, and produce virions that are not as effectively camouflaged. Consequently, these virions will retain slightly higher infectivity. Importantly, relative to SCmPEG differences were not as readily noted with SVAmPEG. This is likely the result of its prolonged hydrolysis-half life allowing more mPEG grafting, thus, partially overcoming the effects of self-exclusion with longer polymers. This is particularly evident as no significant differences were found between the 2 and 5 kDa polymers, but differences were found between the short (2 and 5 kDa) polymers and the longer 20 kDa polymer. Thus, taken together with the findings on linker chemistry (see section 4.1.2), these results suggest that short polymer length (*i.e.* 2 kDa) in combination with the SVA linker chemistry will most dramatically inhibit RSV infection.

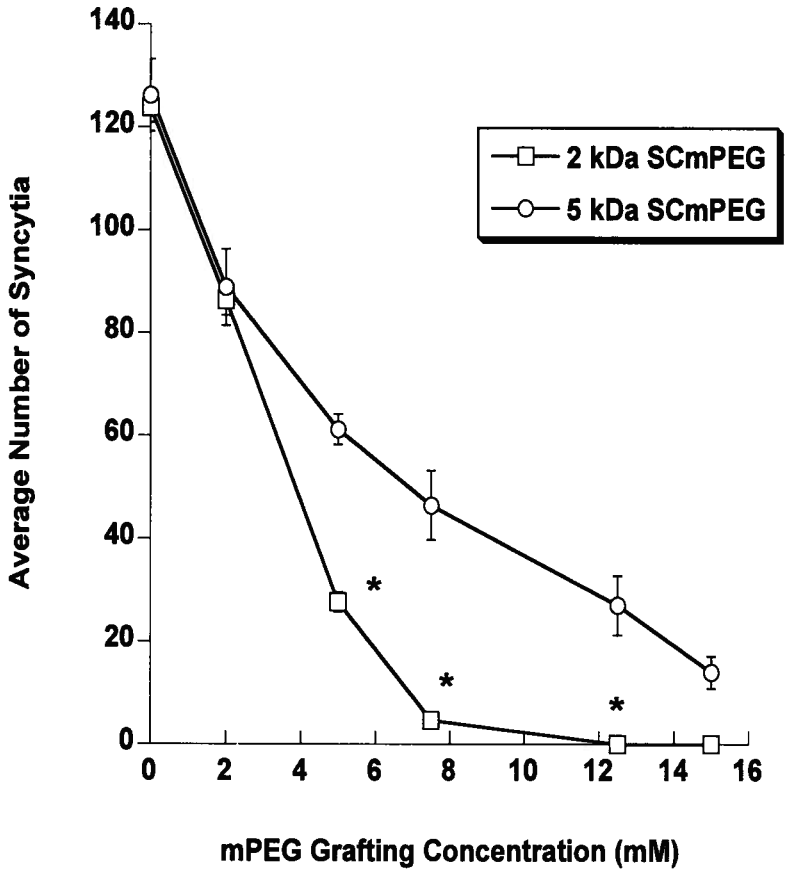


Figure 4.5. Modification of RSV with 2 kDa SCmPEG Provided Enhanced Protection Relative to 5 kDa SCmPEG. RSV was modified with 2 or 5 kDa SCmPEG and then used to challenge Vero cells in a plaque assay. Both mPEG species significantly decrease RSV infection in a dose-dependent manner ($p<0.002$) with 2 kDa SCmPEG providing significantly better protection than 5kDa SCmPEG ($p<0.012$).
Values shown are mean $\pm$ SE for three separate experiments performed in duplicate.
* indicates significantly different 5 kDa SCmPEG ($p<0.012$).

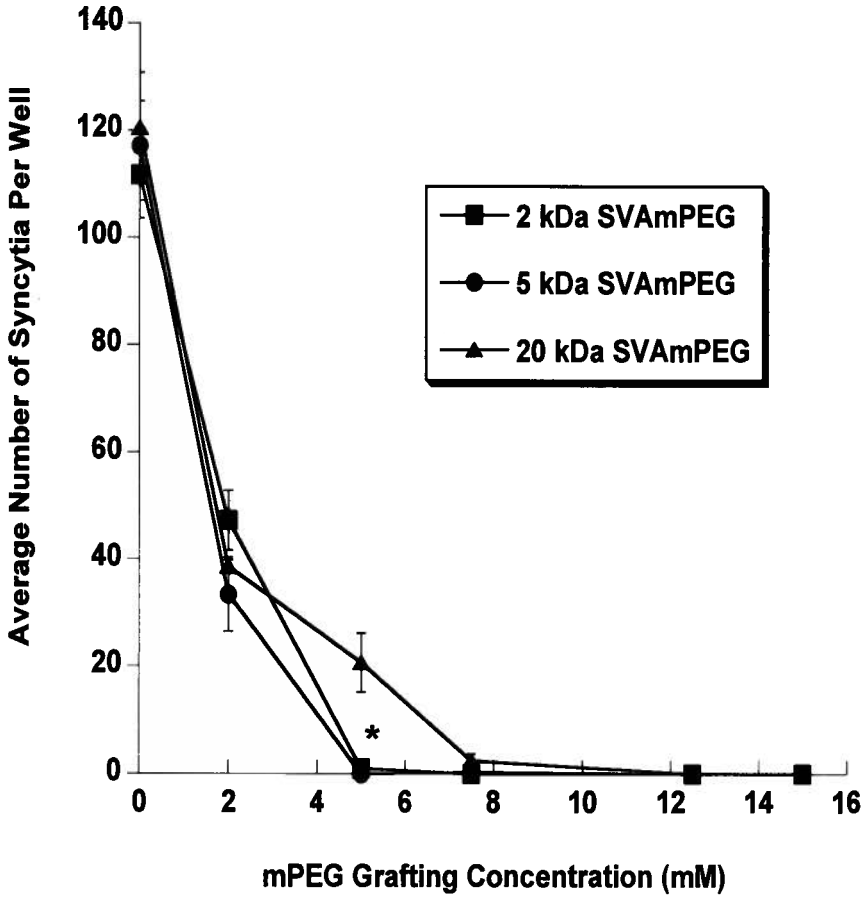


Figure 4.6. Modification of RSV with 2, 5, or 20 kDa SVAmPEG Dramatically Reduces RSV Infection. RSV was modified with 2, 5, or 20 kDa SVAmPEG and then used to challenge Vero cells in a plaque assay. All three mPEG species significantly decreased RSV infection in a dose-dependent manner ($p<0.001$), and with the 2 and 5 kDa species showing significantly improved protection at 5 mM grafting concentration ($p<0.025$).
Values shown are mean $\pm$ SE for three separate experiments performed in duplicate.
* indicates significantly different from 20 kDa SVAmPEG ($p<0.025$).

Table 4.3. For mPEG-Modification of RSV, Shorter mPEG Polymers Provided Enhanced Protection in Plaque Assay Experiments.

SCmPEG Linker Chemistry: Effect of Polymer Length on the Average Number of Syncytia Per Well and the Percent Reduction						
	mPEG Grafting Concentration (mM)					
Polymer Length	0	2	5	7.5	12.5	15
2 kDa	124 ± 3 (0 %)	*86 ± 3 (30 %)	†28 ± 2 (78 %)	†5 ± 1 (96 %)	†0 ± 0 (100 %)	*0 ± 0 (100 %)
5 kDa	126 ± 7 (0 %)	*89 ± 7 (30 %)	*61 ± 3 (52 %)	*47 ± 7 (63 %)	*27 ± 6 (79 %)	*14 ± 3 (89 %)
SVAmPEG Linker Chemistry: Effect of Polymer Length on the Average Number of Syncytia Per Well and the Percent Reduction						
2 kDa	112 ± 5 (0 %)	*47 ± 6 (58 %)	§1 ± 0.7 (99 %)	*0 ± 0 (100 %)	*0 ± 0 (100 %)	*0 ± 0 (100 %)
5 kDa	117 ± 14 (0 %)	*33 ± 7 (72 %)	§0 ± 0 (100 %)	*0.3 ± 0.3 (99 %)	*0 ± 0 (100 %)	*0 ± 0 (100 %)
20 kDa	121 ± 5 (0 %)	*39 ± 1 (68 %)	*21 ± 5 (83 %)	*3 ± 1 (98 %)	*0 ± 0 (100 %)	*0 ± 0 (100 %)

Values represent the number of syncytia per well at five days post-infection, and are expressed as mean ± standard error. Values represent averages of three experiments performed in duplicate. Values in brackets represent the percent reduction relative to the 0 mM control.
* significantly different from the 0 mM control ($p<0.025$).
† significantly different from 5 kDa SCmPEG ($p<0.012$) and the 0 mM control ($p<0.025$).
§ significantly different from 20 kDa SVAmPEG and the 0 mM control ($p<0.025$).

4.2 LOG SCALE GFP-RSV EXPERIMENTS: mPEG-MODIFICATION OF GFP-RSV OVER LOG SCALE DOSES OF VIRUS

As the natural RSV inoculation dose varies widely, we sought to evaluate the efficacy of mPEG-mediated viral inhibition over a broad range of virus doses. To test the efficacy of mPEG-modification at high virus doses, we chose MOI of 0.5 and 0.1, and to test more physiological ranges of virus infection, we chose of MOI of 0.05 and 0.01. Furthermore, we sought to evaluate the effect of polymer length over these virus doses and, therefore, modified GFP-RSV with 2 or 20 kDa SVAmPEG and challenged non-polarized HeLa cells. mPEG grafting concentrations included 0, 2, 5, 7.5, 12.5, and 15 mM, and the percentage of infected cells was determined by flow cytometry at 12, 24, 48, 72, and 96 hours post-infection. Importantly, using GFP-RSV we were able to quantify infection over time, and could therefore study both the initial reduction in infection and subsequent viral proliferation.

4.2.1 Log Scale GFP-RSV Experiments: Characterization of GFP-RSV

Prior to performing log scale experiments, we characterized GFP-RSV infection of HeLa cells at MOI of 0.5, 0.1, 0.05, and 0.01, from 0 to 48 hours. Shown in Figure 4.7 are the growth curves for GFP-RSV, and in Figure 4.8 are images of GFP-RSV infected cells followed over time. Examining the growth curves, the percentage of infected cells increased significantly by 2 hours for MOI 0.5, 4 hours for MOI 0.1, 8 hours for MOI 0.05, and 36 hours for MOI 0.01 ($p<0.001$). For all MOI evaluated, the number of infected cells increased over time. Furthermore, by 8 hours post-infection all curves were distinct from each other except the curve for MOI 0.01, which did not become significantly different from the background (MOI 0) until 36 hours post-infection.

For MOI of 0.5, 0.1, and 0.05, the growth curves have a plateau phase from 8 to 20 hours, which may indicate the establishment of a primary infection. This plateau is then followed by a dramatic increase in the number of infected cells that likely represents the establishment of a secondary infection by progeny virus. For the MOI of 0.01, the low level of infection obscured the expected plateau phase. Thus, in subsequent log scale virus dose experiments, particularly at low virus doses, all MOI were followed out to 96 hours to ensure that any reduction in infection with mPEG-modification could be observed.

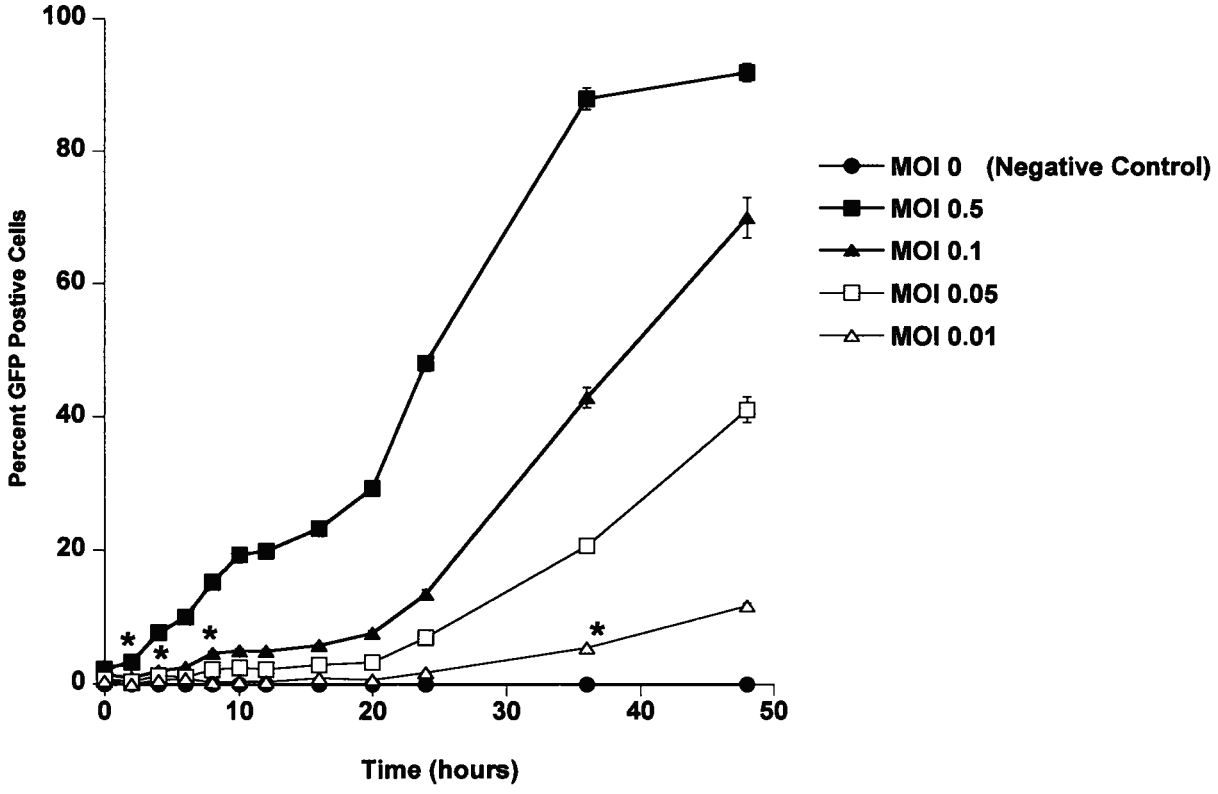


Figure 4.7. GFP-RSV Growth Curves. HeLa cells were challenged with GFP-RSV at an MOI of 0.5, 0.1, 0.05, 0.01, and 0, and the percentage of infected cells was determined by flow cytometry. Infected cells were counted at time points from 2 to 48 hours.
Values expressed are mean $\pm$ SE of two experiments performed in quadruplicate.
* indicates significantly different from MOI 0 from the designated time onwards ($p<0.001$).

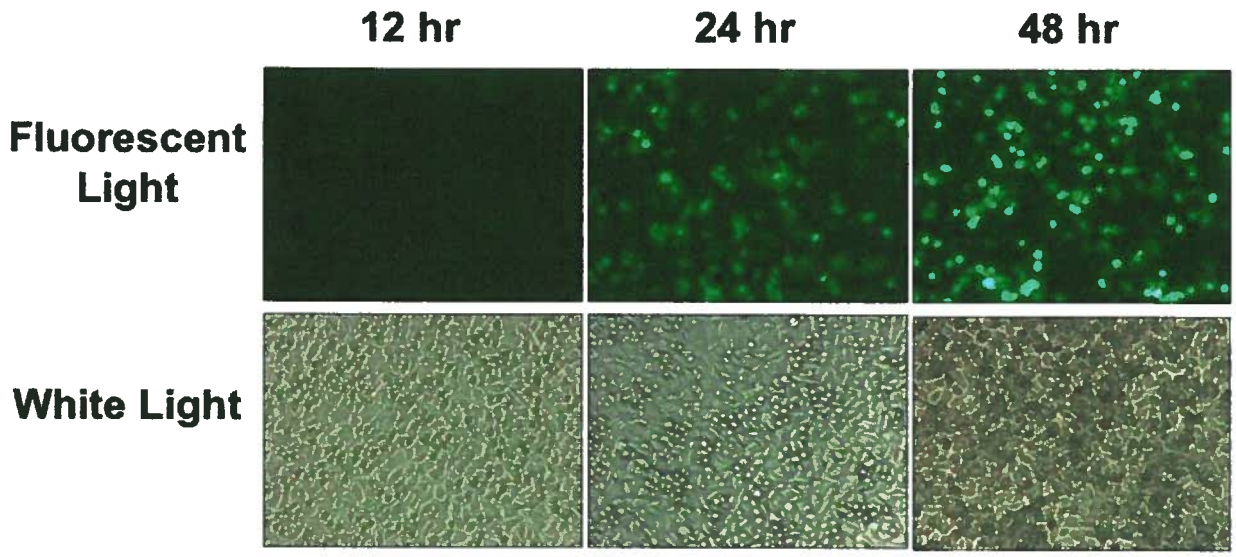


Figure 4.8. GFP-RSV Infected Cells Examined Under Both Fluorescent and White Light. HeLa cells were infected at an MOI of 0.5 and were followed for 48 hours. Representative images of two experiments performed in quadruplicate were taken at 100X magnification under both white and fluorescent light.

4.2.2 Effect of Polymer Length and Grafting Density Over Log Scale Virus Doses

To test the upper boundaries of the efficacy of mPEG-modification of RSV, we evaluated virus doses of MOI 0.5 and 0.1. Subsequently, we then sought to evaluate more "physiological" or natural inoculation doses and examined MOI of 0.05 and 0.01. At all MOI examined, using either 2 or 20 kDa SVAmPEG, mPEG grafting decreased the percentage of infected cells in a dose dependent manner ($p<0.045$). The results for MOI of 0.5 and 0.1 are shown in Figure 4.9, and the results for MOI of 0.05 and 0.01 are show in Figure 4.10. Results are also displayed as percent reduction in infection in Table 4.4.

Examining the high MOI, at an MOI of 0.5, grafting of 2 mM mPEG at either polymer length did not significantly decrease the level of infection. However, at an MOI of 0.1, grafting of 2 mM mPEG significantly decreased the level of infection with both polymer lengths. For example, 2 kDa mPEG decreased infection by 41% at 48 hours, while 20 kDa mPEG reduced infection by 25% (see Table 4.4). Importantly at this 2 mM concentration, grafting of the 2 kDa mPEG resulted in a significantly greater reduction in infection than grafting of 20 kDa mPEG ($p<0.002$) from 48 hours onwards. Next, at the 5 mM grafting concentration and an MOI of 0.5, both 2 and 20 kDa mPEG significantly decreased the level of infection, and no significant differences were found between the polymer lengths. At an MOI of 0.1, grafting of 5 mM mPEG also significantly reduced the level of infection ($p<0.006$) with decreases of greater than 48% and no significant differences between the 2 and 20 kDa polymers.

Focusing on the 7.5 mM grafting concentration with both polymer lengths, infection was dramatically decreased with a greater than 70% and 89% reduction at MOI of 0.5 and 0.1, respectively. Furthermore, at both MOI the level of infection was significantly lower with 2 kDa mPEG compared to 20 kDa mPEG ($p<0.006$) from 48 hours onwards. For example, at an MOI of 0.5 at 72 hours post-infection, 7.5 mM 2 kDa mPEG grafting decreased infection by 91% while 20 kDa mPEG decreased infection by 71.7%. Importantly, grafting of 7.5 mM mPEG also

resulted in the most dramatic decrease in infection per unit (mM) of mPEG. At higher grafting concentrations of 12.5 and 15 mM, both polymer lengths almost completely inhibited infection.

Considering the "physiological" MOI of 0.05 and 0.01 (see Figure 4.10), we found that 2 mM and higher grafting concentrations significantly decreased the level of infection relative to the 0 mM control ($p<0.007$). Similar to our findings with an MOI of 0.1, at the 2 mM grafting concentration with both MOI of 0.05 and 0.01, grafting of 2 kDa mPEG resulted in a significantly greater inhibition of infection than 20 kDa mPEG grafting ($p<0.007$). This is evident by examining the MOI of 0.01 at 72 hours. At this time point, 2 kDa mPEG grafting decreased infection by 54%, while 20 kDa mPEG grafted decreased infection by 30.4%. At higher grafting concentrations, we observe further decreases in the level of infection; however, there were no significant differences between the 2 and 20 kDa polymers. Importantly, at both MOI of 0.05 and 0.01, grafting of 7.5 mM mPEG resulted in near complete inhibition of viral infection, and at higher concentrations of 12.5 and 15 mM this protective effect was not significantly enhanced. Therefore, across all MOI examined, a grafting concentration of 7.5 mM produced the most dramatic decrease in infection per unit (mM) of mPEG. Furthermore, at all MOI, when mPEG grafting resulted in a significant decrease in infection, the level of infection did not recover to control (0 mM) levels over the 96 hours. This indicates that mPEG grafting was able to dramatically inhibit both the initial viral invasion and subsequent viral proliferation.

In summary, at all virus doses examined both 2 and 20 kDa mPEG-modification of RSV inhibited infection in a dose dependent manner. At all MOI, grafting concentrations of 7.5 mM or higher dramatically decreased viral infection and blunted subsequent proliferation. At high MOI (0.5 and 0.1), with a grafting concentration of 7.5 mM, 2 kDa mPEG more substantially inhibited infection than 20 kDa mPEG, and at low MOI, 2 kDa mPEG similarly provided enhanced protection at the 2 mM grafting concentration. Therefore, our results show that

mPEG-modification is a highly effective strategy for inhibiting RSV infection with grafting of shorter polymers showing improved efficacy.

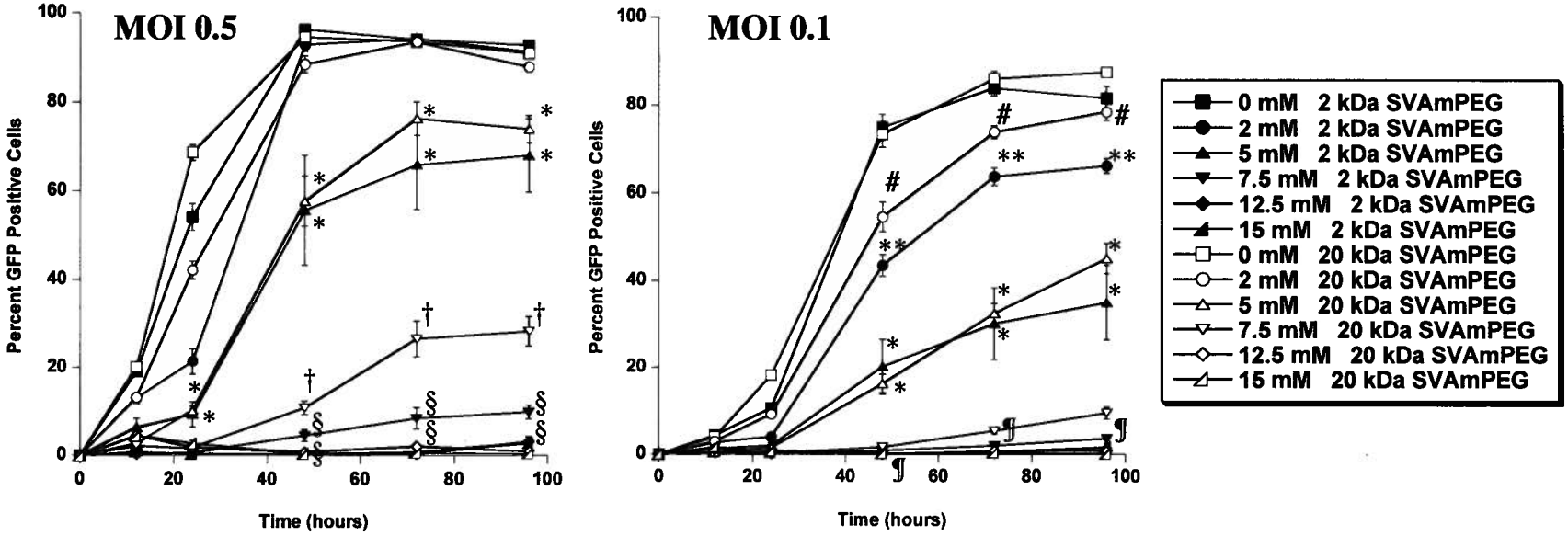


Figure 4.9. Modification of GFP-RSV with 2 kDa mPEG Provided Enhanced Protection Relative to 20 kDa mPEG at 7.5 mM Grafting Concentration and MOI of 0.5 and 0.1. Modification of GFP-RSV with either 2 or 20 kDa SVAmPEG results in an mPEG dose dependent decrease in the percent of cells infected. At a grafting concentration of 7.5 mM, 2 kDa mPEG inhibits RSV infection to a greater extent than 20 kDa mPEG ($P<0.036$).

Values expressed are mean $\pm$ SE of two separate experiments performed in quadruplicate.

* indicates significantly different from 0 mM ($p<0.001$), with no significant differences between 2 and 20 kDa polymers.

† significantly different from all 20 kDa mPEG concentrations ($p<0.006$).

§ significantly different from 0, 2, 5 and 7.5 mM at both the 2 and 20 kDa mPEG concentrations ($p<0.006$).

significantly different from all other 2 and 20 kDa mPEG concentrations ($p<0.006$).

** indicates significantly different from the 0 mM control at the same polymer length and significantly different from the 20 kDa polymer ($p<0.007$).

¶ significantly different from the 20 kDa polymer at 7.5 mM grafting concentration ($p<0.036$).

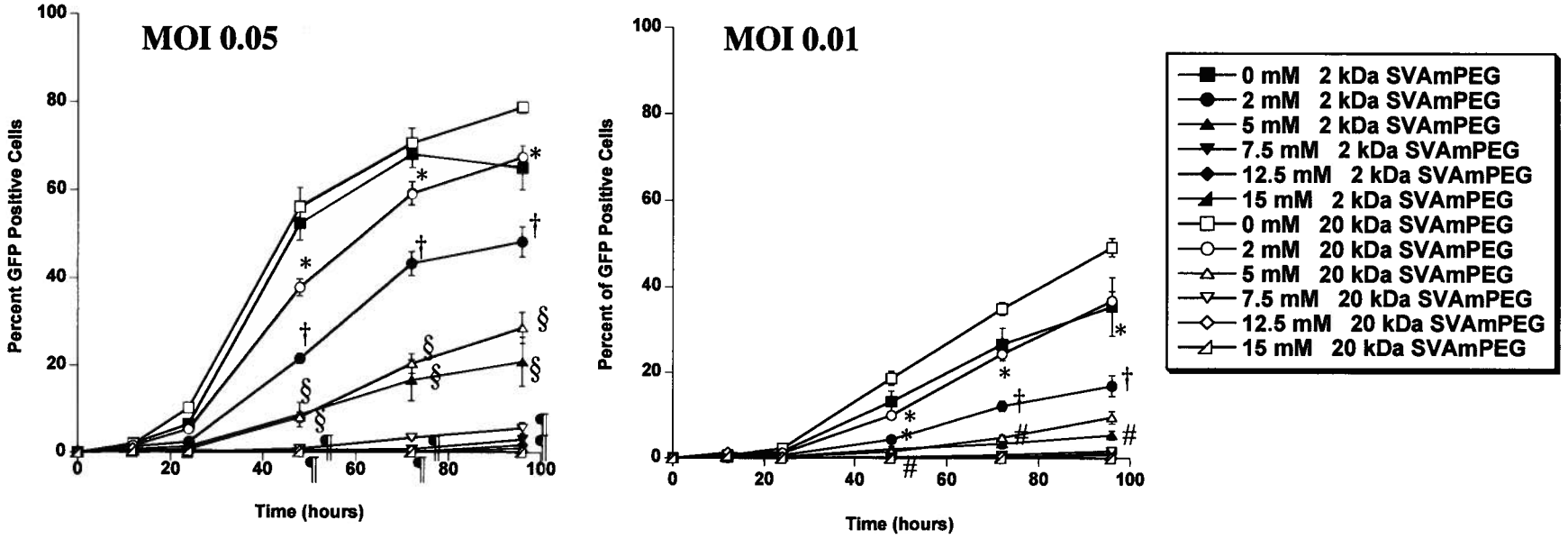


Figure 4.10. Modification of GFP-RSV with 2 kDa mPEG Provided Enhanced Protection Relative to 20 kDa mPEG at 2 mM Grafting Concentration and MOI of 0.05 and 0.01. Modification of GFP-RSV with either 2 or 20 kDa SVAmPEG results in an mPEG dose dependent decrease in the percentage of infected cells. At a grafting concentration of 2 mM, 2 kDa mPEG inhibits RSV infection to a greater extent than 20 kDa mPEG ($p<0.02$).
Values expressed are mean $\pm$ SE of two separate experiments performed in quadruplicate.
* indicates significantly different from all mPEG concentrations with the same polymer length and from 2 kDa mPEG ($p<0.046$).
† significantly different from all mPEG concentrations at the same polymer length and significantly different from 20 kDa mPEG ($p<0.04$).
§ significantly different from 0, 2, 12.5 and 15 mM mPEG concentrations at the same polymer length ($p<0.02$).
¶ significantly different from 0, 2, 5 and 7.5 mM at the same polymer length ($p<0.001$).
# significantly different from 0, 2 and 5 mM at the same polymer length ($p<0.046$).

Table 4.4. Table of the Percent Reduction with mPEG-Modified GFP-RSV Over Log Scale Virus Doses

Comparison of the Percent Reduction for 2 and 20 kDa SVAmPEG-Modified GFP-RSV at Several MOI										
	Time Points									
	12 hours		24 hours		48 hours		72 hours		96 hours	
Polymer Length (kDa)	**2**	**20**	**2**	**20**	**2**	**20**	**2**	**20**	**2**	**20**
mPEG Concentration	MOI 0.5									
0 mM	0	0	0	0	0	0	0	0	0	0
2 mM	31.8	33.9	60.4	38.5	3.6	6.4	0	0.1	1.6	3.4
5 mM	*66.5	*85.7	*82.9	*85.1	*42.2	*39.0	*30.0	*18.5	*26.7	*18.7
7.5 mM	*96.8	*88.5	*98.7	*97.4	†95.3	†88.6	†91.1	†71.7	†89.4	†68.9
12.5 mM	*98.0	*77.0	*99.1	*97.2	*99.6	*99.2	*99.6	*97.8	*96.6	*98.9
15 mM	*95.3	*77.3	*99.3	*96.1	*99.6	*99.6	*99.1	*99.3	*96.9	*99.5
	MOI 0.1									
0 mM	0	0	0	0	0	0	0	0	0	0
2 mM	37.3	*24.8	*61.8	*49.4	†41.9	†25.6	†23.9	†14.1	†18.7	†10.3
5 mM	*70.6	*62.4	*81.0	*91.3	*73.1	*77.8	*64.1	*62.2	*57.1	*48.4
7.5 mM	*92.0	*62.3	*96.7	*99	†98.9	†97.7	†97.6	†93.7	†95.6	†89.1
12.5 mM	*90.4	*69.1	*98.5	*98.4	*99.6	*99.9	*99.7	*99.1	*98.3	*99.1
15 mM	*86.1	*58.6	*95.6	*95.9	*99.7	*99.6	*99.3	*99.5	*98.0	*99.9
	MOI 0.05									
0 mM	0	0	0	0	0	0	0	0	0	0
2 mM	33.9	16.7	*61.9	*47.5	†58.8	†32.7	†36.3	†16.2	†25.7	†14.4
5 mM	*64.0	*48.7	*78.4	*91.8	*83.5	*85.6	*75.6	*71.1	*67.9	*63.6
7.5 mM	*90.7	*45.5	*96.2	*98.7	*98.8	*98.4	*98.7	*95.1	*95.3	*92.8
12.5 mM	*88.2	*44.9	*99.2	*96.2	*99.7	*99.3	*99.7	*99.2	*97.3	*99.6
15 mM	*90.5	*76.9	*100	*95.1	*99.5	*99.6	*99.8	*99.7	*98.4	*99.8
	MOI 0.01									
0 mM	0	0	0	0	0	0	0	0	0	0
2 mM	0	0	*59.2	*47.4	*66.9	*46.5	†54.4	†30.4	†52.2	†25.3
5 mM	0	0	*80.5	*90.2	*84.1	*91.6	*87.2	*86.3	*84.9	*80.7
7.5 mM	0	0	*92.3	*91.6	*99.3	*98.5	*99.1	*97.8	*97.3	*96.6
12.5 mM	0	0	*99.6	*90.7	*98.7	*100	*99.0	*98.9	*96.7	*100
15 mM	0	0	*100	*97	*98.9	*99.4	*98.8	*99.9	*97.8	*100

Values are average percent reduction of two experiments performed in quadruplicate. For all experiments SE was less than 15%.

* indicates significantly different from the 0 mM control ($p<0.045$),

Shaded Light Grey Areas and † indicated significant differences between 2 and 20 kDa mPEG ($p<0.04$) and significantly different from the 0 mM control ($p<0.045$).

4.3 EFFECT OF mPEG-MODIFICATION OF RSV IN POLARIZED MDCK CELLS

As RSV specifically infects polarized epithelial cells *in vivo*, we sought to evaluate mPEG-modification of RSV in the polarized MDCK cell line. For these experiments, we used a high MOI of 8 to overcome the reduced susceptibility of this cell line to RSV. Consequently, GFP-RSV was modified with 2 kDa SVAmPEG at a grafting concentration of 7.5 mM, and then used to challenge the cells. This mPEG linker and concentration were chosen because in combination, they provided optimal protection in both plaque assay and log scale studies. As shown in Figure 4.11, mPEG-modification of GFP-RSV significantly decreased the number of infected cells by greater than 99% ($p<0.001$) at both 24 and 48 hours post-infection. Furthermore, from 24 to 48 hours the number of infected cells in the 0 mM control increased by approximately 100, while in the cells challenged with mPEG-modified virus there was no dramatic increase. This indicates that the virions that managed to infect the cells were not able to generate significant amounts of progeny to overcome the initial reduction in infection. Therefore, given that RSV naturally infects polarized cells in the human airway, our findings strongly suggest that mPEG-modification of the virus will similarly prevent infection *in vivo*.

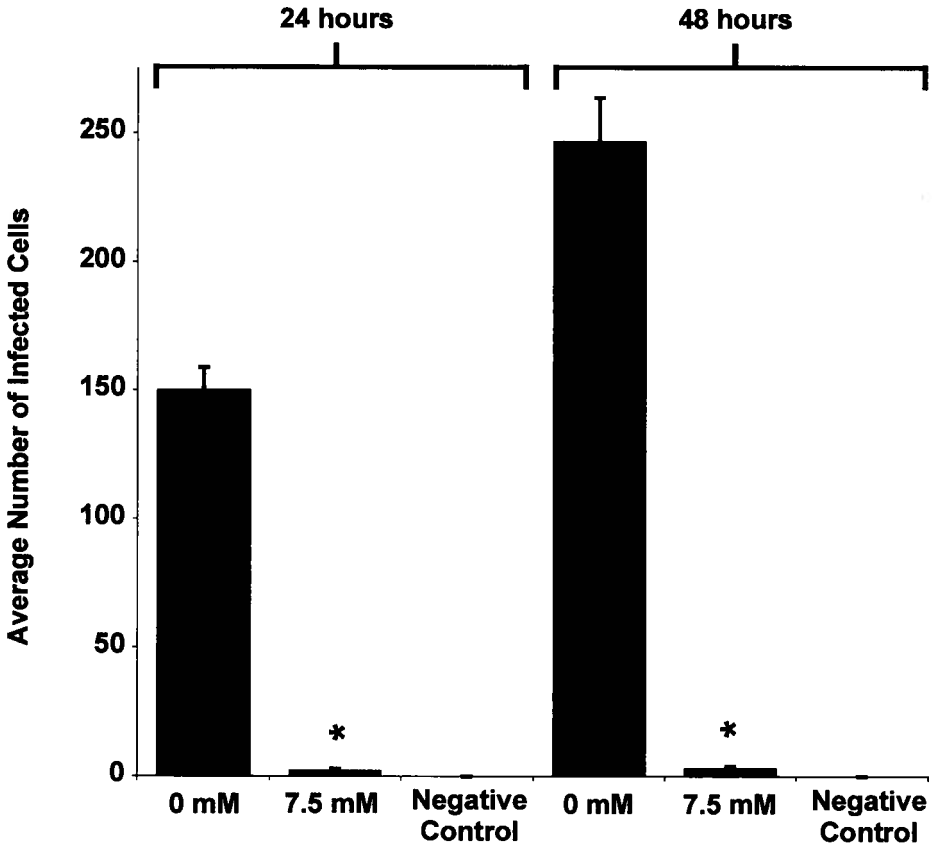


Figure 4.11. 2 kDa SVAmPEG-Modification of GFP-RSV Dramatically Decreases Infection in Polarized MDCK Cells. Polarized MDCK cells were challenged with 2 kDa SVAmPEG modified RSV at a grafting concentration of 7.5 mM. The virus concentration was equal to MOI 8 due to the decreased susceptibility of these cells to RSV.
Values shown are mean $\pm$ SE for two separate experiments performed in quadruplicate.
* indicates significantly different from the 0 mM condition ($p<0.001$).

4.4 EFFECT OF mPEG-MODIFICATION ON RSV VIRION INTEGRITY

Given the high efficacy of mPEG-modification of RSV, we sought to evaluate if mPEG-modification was disrupting the integrity of the RSV virion. GFP-RSV used in the log scale experiments, was modified with 2 kDa SVAmPEG and examined by transmission electron microscopy (TEM).

Shown in Figure 4.12 are TEM images of unmodified (0 mM) and mPEG-modified GFP-RSV. Qualitative analysis of the TEM grids showed no differences in the number of virus particles (data not shown), and as shown in these images, the RSV virion was intact at all mPEG grafting concentrations. Furthermore, both unmodified and modified virus ranged in size from 150 to 300 nm. This is consistent with previous reports on the size of RSV (4), and indicates that mPEG grafting did not dramatically alter the size of the RSV virion.

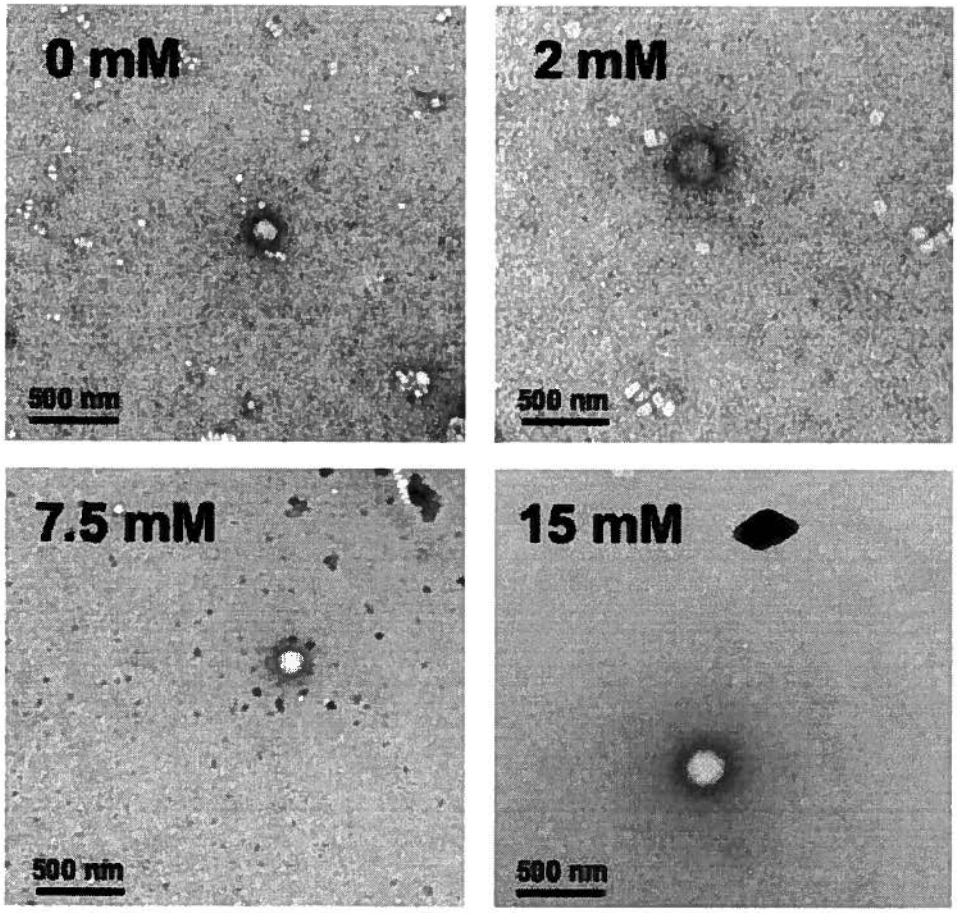


Figure 4.12. 2 kDa SVAmPEG-Modification of GFP-RSV Does Not Disrupt the RSV Virion as Determined by Electron Microscopy. Shown are images of 0, 2, 7.5, and 15 mM mPEG-modified GFP-RSV at 100,000X magnification. These images demonstrate that mPEG grafting does not disrupt the RSV virion and are representative of two experiments performed in duplicate.

4.5 SUMMARY OF mPEG-MODIFICATION OF RSV

In all experiments, mPEG-modification of RSV decreased RSV infection in a dose-dependent manner. In plaque assay experiments, SVA linker chemistry and short polymer length (2 kDa) were shown to provide enhanced inhibition of RSV infection. In the context of log scale doses of RSV, we compared the efficacy of 2 and 20 kDa SVAmPEG. Our results show that at low to moderate grafting concentrations, 2 kDa mPEG provided enhanced protection compared to 20 kDa mPEG. In contrast, at high grafting concentrations, both polymers provided similar levels of protection.

Importantly, these studies demonstrated that even with high virus doses (*i.e.* MOI 0.5 and 0.1), mPEG grafting concentrations of 7.5 mM or greater resulted in significant protection from RSV. Furthermore, mPEG-modification both dramatically decreased the initial infection and also severely limited or slowed subsequent viral proliferation over 96 hours. At more physiologic virus concentrations (*i.e.* MOI 0.05, and 0.01), mPEG grafting at 7.5 mM or greater resulted in near complete abrogation of RSV infection, and subsequent proliferation. To further gain insight into whether mPEG-modification would be effective *in vivo,* we evaluated mPEG-modification of RSV in polarized MDCK cells. In this polarized cell line, we found that mPEG-modification of the virus dramatically decreased infection, and similar to our findings in non-polarized cell lines also significantly blunted from proliferation. Lastly, to ensure that PEG-modification was not disrupting the RSV virion, we performed TEM studies and found that PEGylation did not alter the size or morphology of the virus. In summary, mPEG-modification of RSV is a highly effective strategy for preventing viral infection, and this was consistent over a broad range of virus doses and in both non-polarized and polarized cells. Therefore, future experiments should be conducted using *in vivo* models to further explore the use of this technology in both blood banking and vaccine strategies.

CHAPTER 5: RESULTS: mPEG-MODIFICATION OF HOST CELLS

5.0 OVERVIEW

While direct modification of viruses may be possible in some situations (*i.e.* blood banking or vaccine development), it will not be possible with regards to respiratory virus infections (*i.e.* RSV) acquired via normal modes of transmission *(i.e.* accidental self-inoculation with contaminated secretions). The more practical approach to preventing RSV and respiratory virus infections is to modify the host cell rendering them resistant to viral invasion. To achieve this goal, we have evaluated the hypothesis that normal RSV infection can be prevented or attenuated by covalent grafting of mPEG to the host cell surface (see Figure 5.1). As detailed below, we show that mPEG-modification of the host cell with long chain polymers is a highly effective strategy for preventing RSV infection.

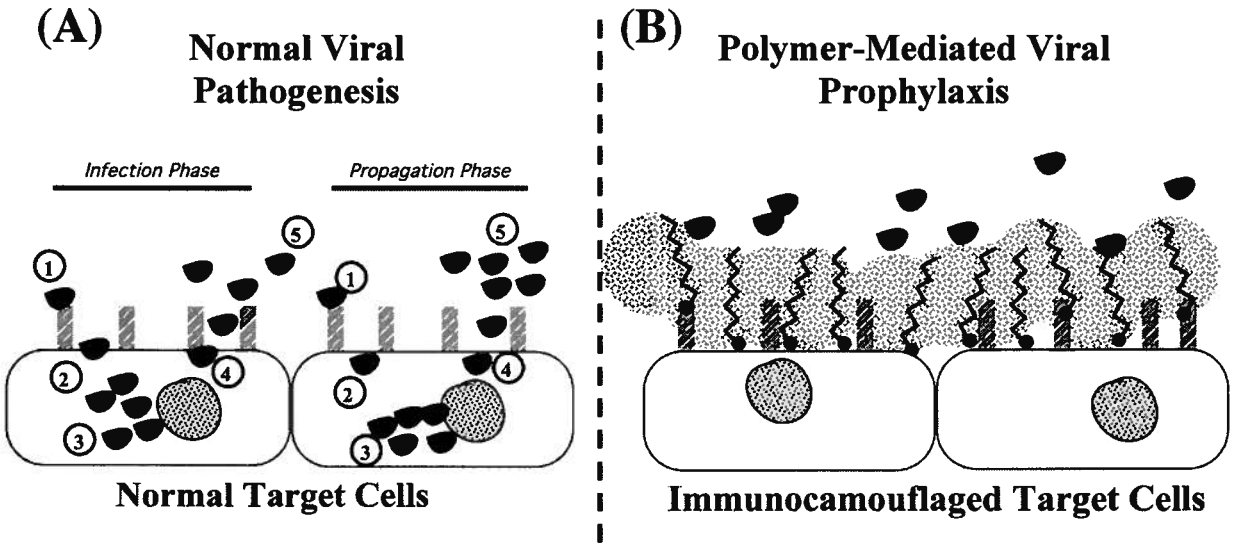


Figure 5.1. Schematic of mPEG-Modified Host Cell Hypothesis. Viral pathogenesis **(A)**: RSV recognizes and binds to the cell surface receptor (1), fuses with the cell membrane and is internalized (2), undergoes multiple rounds of replication (3), progeny virus buds from the cell surface (4) into the extracellular environment (5) whereupon it infects new host cells and the cycle is repeated. **(B)** Covalent grafting of mPEG directly to the host cell surface prevents viral interaction with the surface receptor and the resulting infection.

5.1 FLUORESCEIN-mPEG GRAFTING TO HELA CELLS DEMONSTRATES CELL SURFACE MODIFICATION

To ensure that incubation of cell monolayers with activated mPEG resulted in cell surface modification, we subjected HeLa cells to mPEG-modification with fluorescein-label mPEG. As shown in Figure 5.2, microscopic visualization of mPEG-modified cells under white light shows that at 0, 2, and 7.5 mM grafting concentrations the cells had normal morphology. At the 15 mM grafting concentration, the cells had altered morphology, and it appears that mPEG-modification was toxic to the cells. However, as shown in the lower portion of Figure 5.2, this was not observed with 20 kDa mPEG modification of the cells. As well, mPEG-modification of the cells with 2 kDa mPEG similarly did not result in altered cell morphology (data not shown) (see cell viability section 5.4 or discussion section 6.2.1 for more discussion).

Under fluorescent light, mPEG-modified cells fluoresced green indicating that mPEG had grafted to the cell surface. Importantly, the intensity of the fluorescence increased with grafting concentration indicating that mPEG was grafting to the cell surface in a dose-dependent fashion. As noted in the methods, cells were modified with a mixture of 5% fluorescein-labeled and 95% unlabeled mPEG. Thus, while the signal at the 2 mM grafting concentration was relatively weak, it is representative of only 5% of the total amount of mPEG grafted to the cell surface. Extending this to the 7.5 and 15 mM concentrations, it is likely that the surface proteins on these cells are heavily modified. Furthermore, at all grafting concentrations, the membrane fluorescence was uniform suggesting that global camouflage of the apical cell surface was achieved.

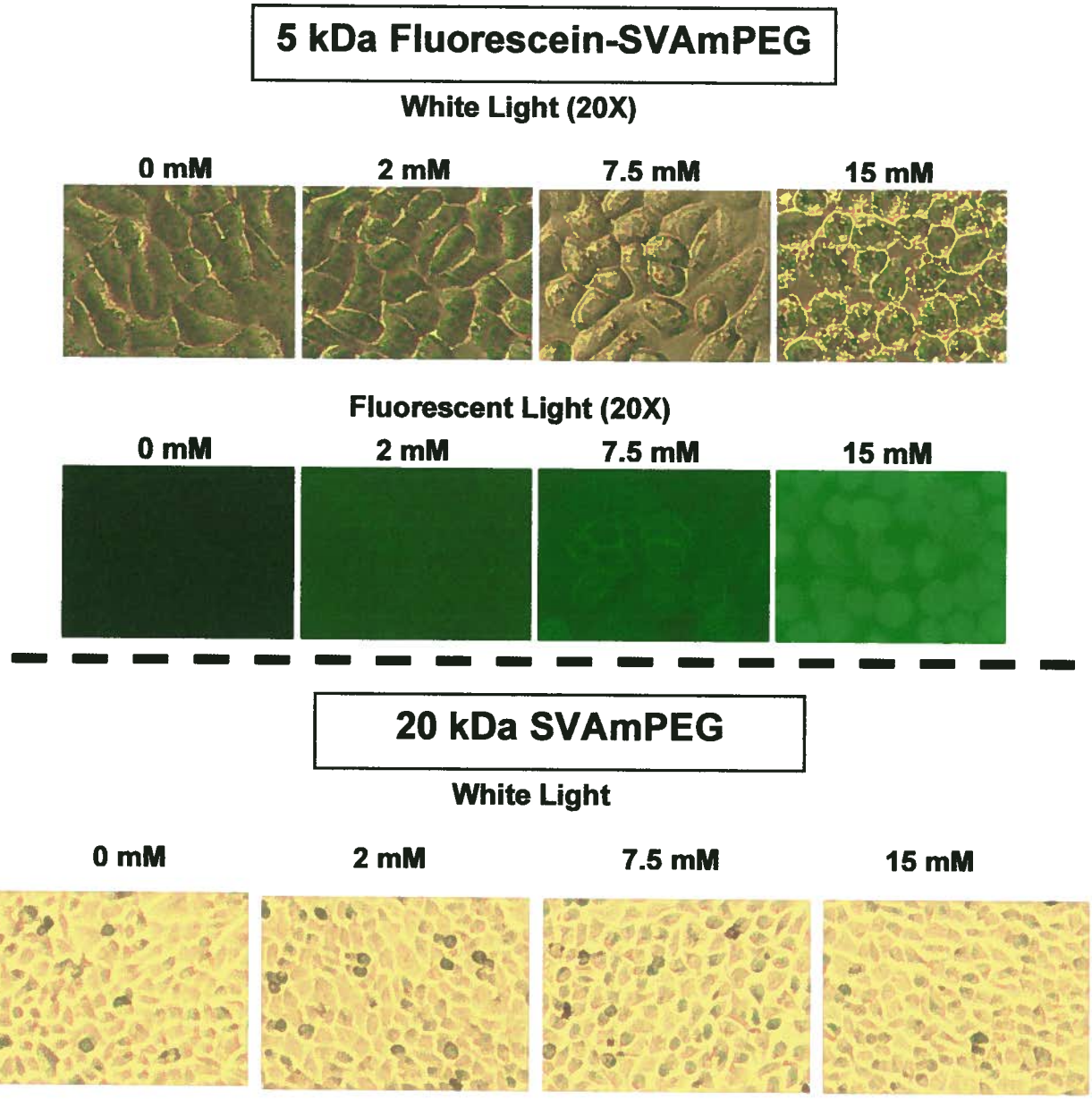


Figure 5.2. Fluorescein Labeled- 5 kDa SVAmPEG and 20 kDa SVAmPEG Modification of HeLa Cells Shows that mPEG Binds to the Cell Surface and Appears to Induce Toxicity at the 5 kDa Length But Not the 20 kDa Length. **(A)** Images of cells under white and fluorescent light that were modified with a mixture of 5 kDa SVAmPEG containing 5% fluorescein-labeled mPEG and 95% unlabeled mPEG. **(B)** Images of cells under white light that were modified with 20 kDa SVAmPEG. Unlike cells modified with 5 kDa mPEG, cells modified with 20 kDa mPEG do not have altered morphology and do not show signs of toxicity. Cells were modified under standard conditions (30 minutes at room temperature in pH 7.8, DMEM). Images are representative of two experiments performed in duplicate.

5.2 LOG SCALE GFP-RSV CHALLENGE OF mPEG-MODIFIED CELLS: EFFECT OF POLYMER LENGTH AND GRAFTING DENSITY OVER LOG SCALE INCREASES IN VIRUS DOSE

To assess the effect of host cell modification against RSV infection, we modified host cells with 2 or 20 kDa SVAmPEG and challenged them with log scale doses of GFP-RSV. Consistent with log scale studies on mPEG-modified virus (see sections 4.1 and 4.2), we used viral challenge doses of MOI 0.5, 0.1, 0.05, and 0.01, and followed infection from 12 to 96 hours. Importantly, this broad range of challenge doses was chosen to test the upper boundaries of the efficacy of host cell modification (MOI 0.5 and 0.1) and to also evaluate more "physiological" or natural inoculation doses of RSV (MOI 0.05 and 0.01). The SVA linker chemistry was chosen based on its enhanced efficacy in the context of virus modification (see section 4.1.3), and both the 2 and 20 kDa polymer lengths were used to determine the effect of polymer length. The results for MOI of 0.5 and 0.1 are shown in Figure 5.3, and the results for MOI of 0.05 and 0.01 are shown in Figure 5.4. The results are also expressed as a percent reduction of infection in Table 5.1.

Focusing on the high MOI of 0.5 and 0.1, we found that at all 2 kDa mPEG grafting concentrations there was no significant decrease in the level of infection relative to the 0 mM control. However, with 20 kDa mPEG grafting to the host cell, RSV infection was decreased in a dose dependent manner ($p<0.038$). This is in dramatic contrast to our results with mPEG-modification of RSV. Focusing on the different grafting concentrations for 20 kDa mPEG, at both MOI, a grafting concentration of 2 mM did not significantly decrease the level of infection. In contrast, the 5 mM grafting concentration at both MOI caused a significant decrease in the level of infection ($p<0.03$). For example, at an MOI of 0.5, grafting of 5 mM, 20 kDa mPEG reduced infection by 20.6% at 48 hours, while for an MOI of 0.1 there was a reduction of 58.8%.

Examining the 7.5 mM grafting concentration at the MOI of 0.5, there is a reduction of 45% at 48 hours, while at an MOI of 0.1 there is a reduction of 85.1%. Importantly, at an MOI of 0.1, 7.5 mM mPEG grafting decreased infection by more than 65% at all times.

Lastly, with the high grafting concentrations of 12.5 and 15 mM, at both MOI there was no significant difference in the level of protection conferred by these concentrations. At an MOI of 0.5, these high grafting concentrations provided the most dramatic reduction, decreasing infection by greater than 70% at all times. Considering the MOI of 0.1, these concentrations almost completely abolished infection resulting in a greater than 91% decrease at all time points.

Examining the more physiological MOI of 0.05 and 0.01, we again found that 2 kDa mPEG grafting did not significantly decrease infection at any grafting concentration. However, 20 kDa mPEG grating decreased infection in a dose-dependent manner, with higher mPEG concentrations almost completely abolishing infection ($p<0.03$). First, examining 2 mM, 20 kDa mPEG grafting, at both MOI there was no significant reduction in infection. As with the high MOI, a 5 mM grafting concentration caused a significant decrease in the level of infection. For example, at an MOI of 0.05 at 48 hours post-infection there is a reduction of 60.3%, and at an MOI of 0.01 there is a reduction of 78.2%. With 7.5 mM, 20 kDa mPEG grafting, there is a further decrease in the level of infection with a greater than 65% decrease at all time points and with both MOI. Importantly, this 7.5 mM grafting concentration is the lowest mPEG dose that results in the most dramatic reduction in infection.

Lastly, focusing on the 12.5 and 15 mM grafting concentrations, at both MOI there is no significant difference between these concentrations. Furthermore, both concentrations almost completely abrogated infection, resulting in a greater than 85% reduction that often approached 100% depending on the time point examined. Importantly, at all MOI and all time points, when mPEG grafting resulted in a significant decrease in infection, the level of infection was not able to recover to control (0 mM) levels. This suggests that mPEG grafting to the host cells both

dramatically inhibited the initial viral invasion and also prevented the spread of progeny virus. This is not surprising as progeny virus that were released into the media would encounter host cells that had mPEG grafted to the cell surface. Moreover, as these low MOI approximate a natural inoculation dose, our findings indicate that mPEG-modification of the host cells would be able to reduce the level of infection or completely prevent infection *in vivo*.

In summary, 2 kDa mPEG grafting to the host cells does not prevent RSV infection; however, 20 kDa mPEG grafting inhibits infection in a dose-dependent manner. This is in dramatic contrast to our findings on viral modification, which showed enhanced protection with shorter (2 kDa) polymers. Importantly, at all mPEG grafting concentrations greater than or equal to 5 mM, there was a significant decrease in the level of infection. Furthermore, the level of infection was not able to recover to control levels, indicating that mPEG modification of the host cells prevented the initial viral invasion and also the spread of progeny virus. Considering specific grafting concentrations, at 7.5 mM there was a dramatic decrease in the level of infection at all MOI. Subsequently, at high grafting concentrations of 12.5 and 15 mM, infection was almost completely inhibited. Taken together, these findings indicate that mPEG modification of the host cell with grafting concentrations of 7.5 mM or greater is a highly effective method for limiting both viral invasion and the spread of progeny virus.

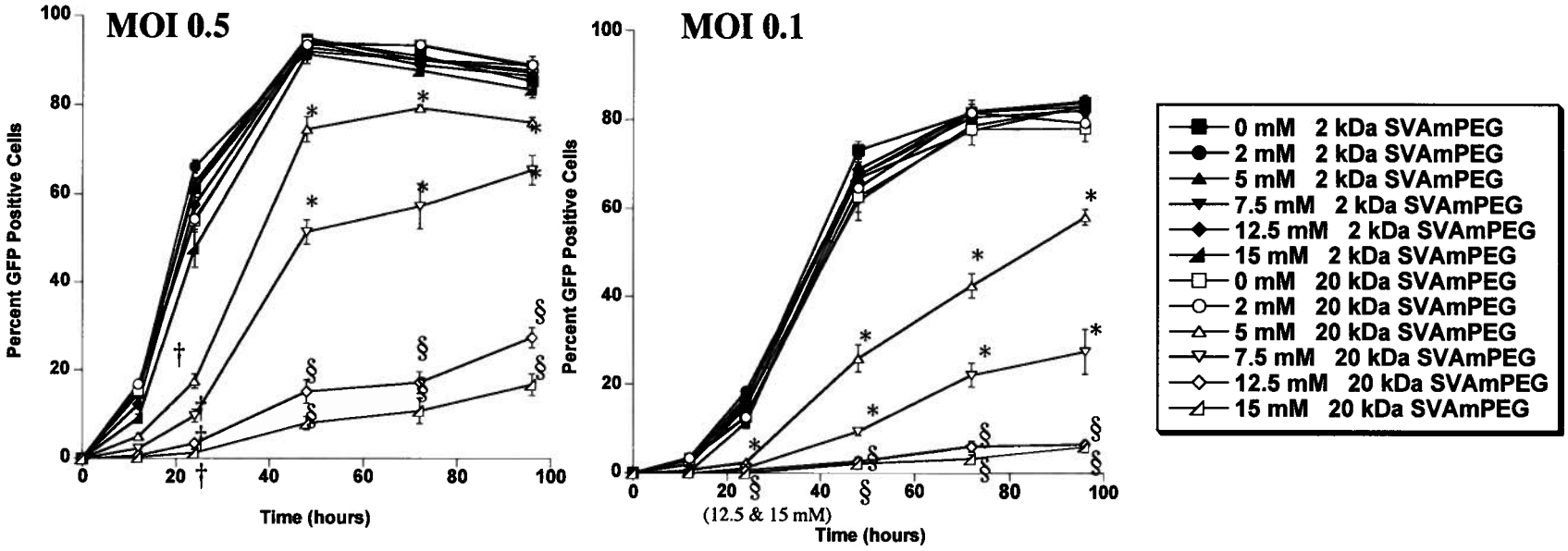


Figure 5.3. 20 kDa SVAmPEG-Modification of HeLa Cells Prevents GFP-RSV Infection in a Dose Dependent Fashion at MOI 0.5 and 0.1. Modification of the host cells with 2 kDa SVAmPEG did not provide significant protection against GFP-RSV challenge. However, modification with 20 kDa SVAmPEG provided protection against RSV infection in a dose dependent manner.
Values expressed are mean ± SE of two separate experiments performed in quadruplicate.
* indicates significantly different from 2 kDa mPEG and from all other mPEG concentrations at the same polymer length ($p<0.038$).
† indicates significantly different from 2 kDa mPEG ($p<0.001$), and 0 and 2 mM at the same polymer length ($p<0.006$).
§ indicates significantly different from 2 kDa mPEG, and 0, 2, 5, and 7.5 mM at the same polymer length ($p<0.035$).

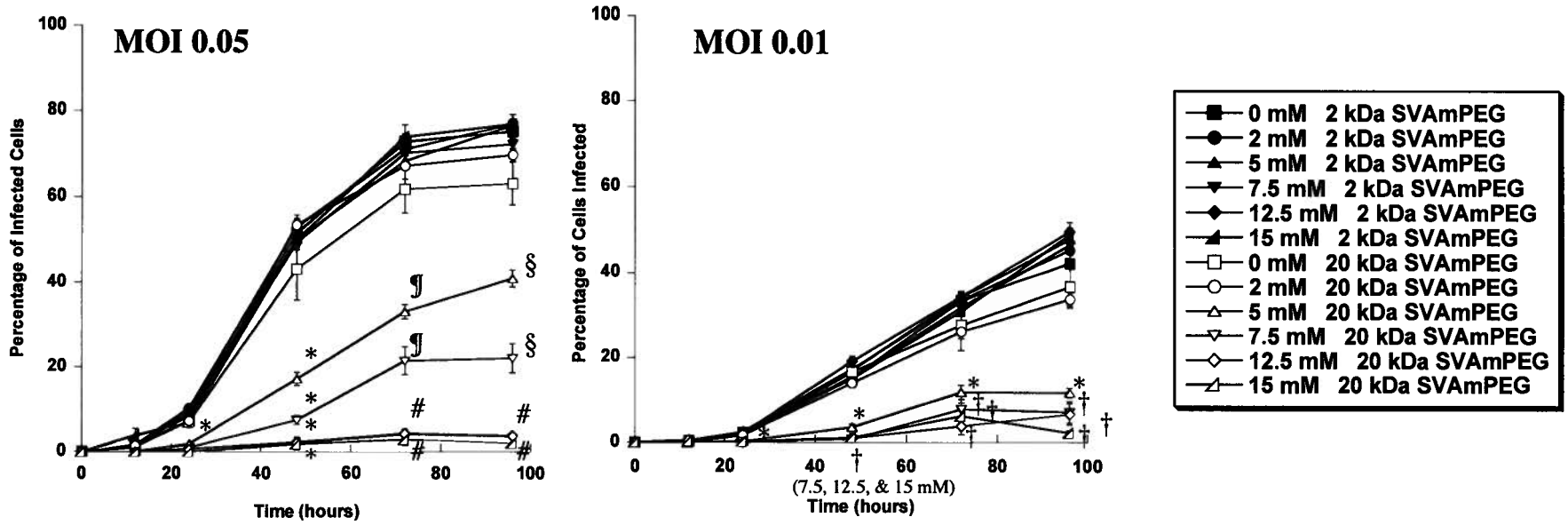


Figure 5.4. 20 kDa SVAmPEG-Modification of HeLa Cells Prevents GFP-RSV Infection in a Dose Dependent Fashion at MOI 0.05 and 0.01. Modification of the host cells with 2 kDa SVAmPEG did not provide significant protection against GFP-RSV challenge. In contrast, modification with 20 kDa SVAmPEG provided protection against RSV infection in a dose-dependent manner.
Values expressed are mean ± SE of two separate experiments performed in quadruplicate.
* indicates significantly different from all 2 kDa mPEG grafting concentrations, and 0 and 2 mM 20 kDa SVAmPEG ($p<0.03$).
† indicates significantly different from all 2 kDa mPEG grafting concentrations, and 0, 2, and 5 mM 20 kDa SVAmPEG ($p<0.003$).
¶ indicates significantly different from all 2 kDa mPEG grafting concentrations, and 0, 2, 12.5, and 15 mM 20 kDa SVAmPEG ($p<0.01$).
§ indicates significantly different from all 2 kDa and 20 kDa mPEG grafting concentrations ($p<0.003$).
# indicates significantly different from all 2 kDa mPEG grafting concentrations, and 0, 2, 5, and 7.5 mM 20 kDa SVAmPEG ($p<0.001$).

Table 5.1. Regions in Log Scale mPEG-Modified Host Cell Studies in which 20 kDa mPEG Provides Significantly Greater Protection than 2 kDa mPEG.

Comparison of the Percent Reduction for 2 and 20 kDa SVAmPEG-Modified HeLa Cells Challenged with GFP-RSV at Several MOI										
	Time Points									
	12 hours		24 hours		48 hours		72 hours		96 hours	
Polymer Length (kDa)	**2**	**20**	**2**	**20**	**2**	**20**	**2**	**20**	**2**	**20**
mPEG Concentration	MOI 0.5									
0 mM	0	0	0	0	0	0	0	0	0	0
2 mM	0	0	0	0	2.8	0.5	0.8	0	0	0
5 mM	0	*67.4	0	*67.6	0	*20.6	0.9	*15.0	0	*14.1
7.5 mM	0.1	*85.0	0.1	*82.1	1.8	*45.3	0.7	*38.6	0	*26.2
12.5 mM	16.7	*95.5	6.2	*93.6	0.5	*83.4	2.2	*81.6	0	*69.1
15 mM	20.1	*97.5	22.5	*97.6	3.4	*91.4	3.5	*88.4	2.2	*81.1
	MOI 0.1									
0 mM	0	0	0	0	0	0	0	0	0	0
2 mM	0	0	0	0	7.6	0	1.2	0	2.1	0
5 mM	0	*77.3	0	*81.2	8.5	*58.8	4.8	*45.5	0.7	*25.6
7.5 mM	0	*100	4.1	*91.1	7.4	*85.1	3.4	*71.7	1.0	*64.9
12.5 mM	10.6	*99.3	7.7	*95.3	5.9	*95.8	0	*92.3	0	*91.7
15 mM	12.7	*95.2	29.7	*100	7.8	*96.5	0	*95.8	1.0	*92.4
	MOI 0.05									
0 mM	0	0	0	0	0	0	0	0	0	0
2 mM	0	0	0	0	0	0	2.4	0	0	0
5 mM	0	*96.6	0	*74.3	2.7	*60.3	6.2	*46.4	0	*35.1
7.5 mM	0	*100	5.1	*86.7	4.2	*82.8	3.7	*65.3	3.8	*65.0
12.5 mM	0	*100	6.5	*90.9	0	*94.9	0.2	*93.0	0	*93.9
15 mM	0	*100	20.0	*100	3.1	*95.4	0	*95.0	0	*96.7
	MOI 0.01									
0 mM	0	0	0	0	0	0	0	0	0	0
2 mM	0	0	0	0	0	15.9	0	5.6	0	7.8
5 mM	0	0	3.3	0	0	*78.2	0	*57.2	0	*68.1
7.5 mM	0	0	1.2	0	0	*94.7	5.0	*71.6	0	*80.5
12.5 mM	0	0	0	0	0	*93.3	0	*85.9	0	*81.8
15 mM	0	0	8.2	0	1.16	*92.4	7.4	*77.3	0	*93.9

Values are average percent reduction of two experiments performed in quadruplicate. For all experiments SE was less than 15%.

Light Grey Shaded Areas indicate where 20 kDa is significantly different from 2 kDa ($p<0.03$).

* indicates significantly different from the 0 mM control ($p<0.009$) and 2 kDa mPEG ($p<0.03$).

5.3 EFFICACY OF mPEG-MODIFICATION OF POLARIZED MDCK CELLS

The typical RSV *in vitro* culture models often use non-polarized cell lines such as Vero or HeLa cells; however in humans RSV naturally infects polarized airway epithelial cells. Thus, to assess whether mPEG-modification would be efficacious in polarized cells, we evaluated PEGylation of polarized MDCK cells. Based on the results from log scale studies, we compared unmodified cells to cells modified with 7.5 mM, 20 kDa SVAmPEG. This grafting concentration was chosen because it provided dramatic decreases in infection at most MOI examined.

Shown in Figure 5.5 are the results of mPEG-modification of MDCK cells at 24 and 48 hours post-infection. At both time points, mPEG-modification dramatically decreased the level of infection ($p<0.001$), with reductions of 80% and 95% at 24 and 48 hours, respectively. Furthermore, while the number of infected cells doubled between 24 and 48 hours in the 0 mM control, there was no increase in the level of infection with the 7.5 mM grafting concentration. This indicates that mPEG-modification of the host cell surface was able to inhibit infection by progeny virus and prevent the development of a secondary infection. Our findings, therefore, show that mPEG-modification is highly effective in polarized cells and prevents both the initial and secondary infections.

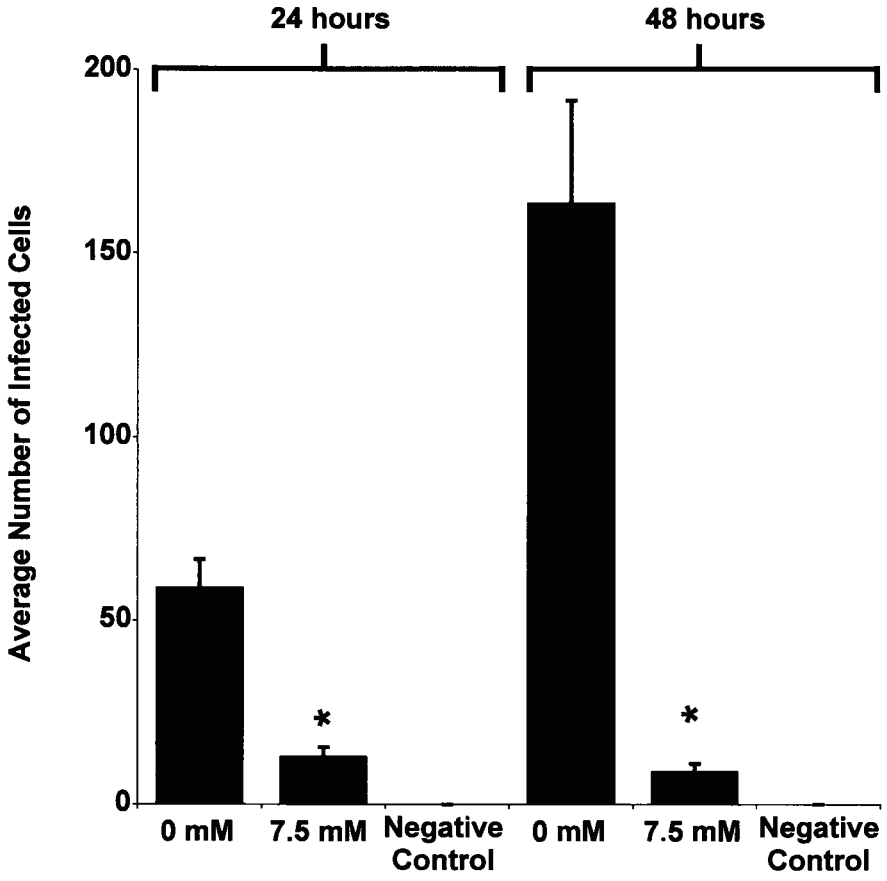


Figure 5.5. 20 kDa SVAmPEG-Modification of Polarized MDCK Cells Significantly Inhibits GFP-RSV Infection. Polarized MDCK cells were modified with 7.5 mM 20 kDa SVAmPEG and then challenged with GFP-RSV at MOI 8. This high MOI was used due to the decreased susceptibility of these cells to RSV. mPEG-modification significantly attenuated RSV infection in polarized cells.
Values expressed are mean $\pm$ SE of two separate experiments performed in quadruplicate.
* indicates significantly different from the 0 mM concentration ($p<0.001$).

5.4 EFFECT OF mPEG-MODIFICATION ON CELL VIABILITY

To ensure that mPEG grafting did not affect cell viability, we performed propidium iodide (PI) staining on dislodged cells, and trypan blue staining on intact cell monolayers. For PI staining, cells were subject to trypsin digest and then analyzed by flow cytometry. However, mPEG-modification of the host cells conferred resistance to trypsin digest, and as a result, mPEG-modified cells required extra pipetting to dislodge and shear the cells from the plate. As shown in Figure 5.6, PI staining showed a significant decrease in cell viability of ~20 and 35% with mPEG grafting concentrations of 7.5 and 15 mM, respectively. However, these findings were not consistent with microscopic analysis of the cells (see Figure 5.7). As shown in Figure 5.7, the mPEG-modified cell layer was intact and mPEG-modification did not result in pronounced changes in cell morphology. Furthermore, as shown in Figure 5.6, the change/loss of viability was the same at both 0 ($\Delta_{0\ hr}$) and 24 (Δ_{24}) hours post-modification. This clearly suggests that the processing (removal of cells from the plate) resulted in cell toxicity as further decreases in viability would be expected over time if mPEG-grafting was harmful. Thus, the findings by PI staining may not have been representative of the actual cell viability and may have been an artifact created by disrupting the cell monolayer. Consequently, to mitigate any effects of dislodging the cells, we performed trypan blue staining on intact cell monolayers and counted the number of dead cells. After counting, to obtain an estimate of the total number of cells, we trypsinized and counted the cells in the unmodified control wells (0 mM condition) and calculated an estimated percent viability for all conditions. Viability was examined at 0, 24, and 48 hours, post-mPEG modification, and both the total number of dead cells and the estimated percent viability are reported in Table 5.2.

By trypan blue staining, at 0 hours post-modification, mPEG grafting of 7.5 and 15 mM resulted in a small but significant increase in the number of dead cells and a corresponding decrease in the percent viability ($p<0.004$). At 24 hours post-mPEG grafting, only the 15 mM

grafting concentration had a small but significant increase in the number of dead cells, with a corresponding decrease in percent viability ($p<0.023$). At 48 hours, there were no significant differences in the number of dead cells or percent viability under all conditions examined.

Considering the findings by trypan blue staining, it is important to note that while these differences are "statistically" significant, they are very small and unlikely to be biologically significant. For example, at all time points viability was greater than 99.8%. Given the dramatic decrease in infection observed in the mPEG-modified cell studies above, this small decrease in percent viability cannot account for mPEG-mediated decrease in RSV infection. Furthermore, by 48 hours post-modification, cell viability under all conditions was not significantly different, indicating that PEGylation of the host cells may be detrimental to cells that are already unhealthy.

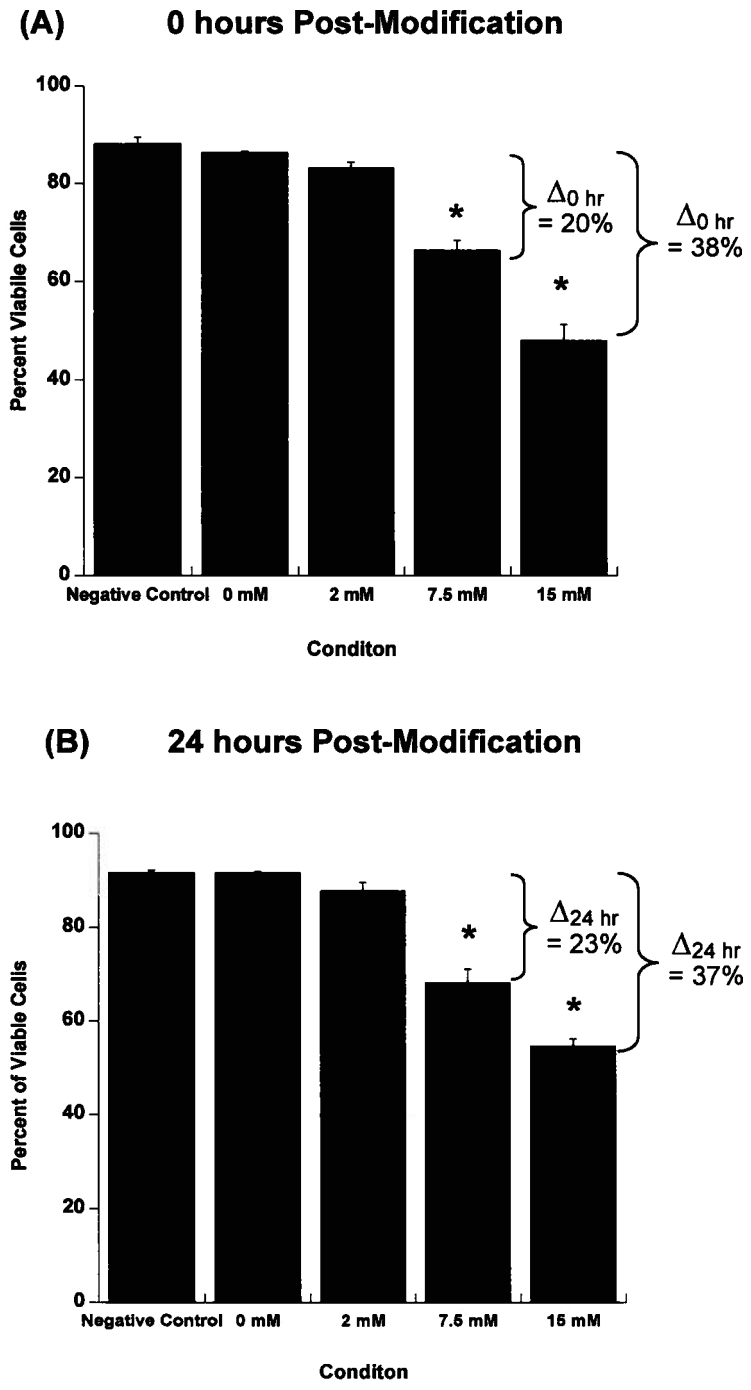


Figure 5.6 Propidium Iodide Staining at 0 and 24 Hours Post- 20 kDa SVAmPEG Modification of HeLa Cells. **(A)** PI Staining at 0 hours post-modification. **(B)** PI staining at 24 hours post-modification. PI staining shows that mPEG-modification appears to have some associated toxicity. However, this may be due to excess handling required to dislodge PEGylated cells as the $\Delta_{0\ hr}$ and $\Delta_{24\ hr}$ were the same. **Note:** PEGylated cells were difficult to dislodge and therefore had to be sheared from the plate via pipetting. Subsequent viability experiments were performed on intact cell monolayers with trypan blue staining. Values expressed are mean $\pm$ SE of two separate experiments performed in quadruplicate.
* indicates significantly different from the 0 mM concentration ($p<0.01$).

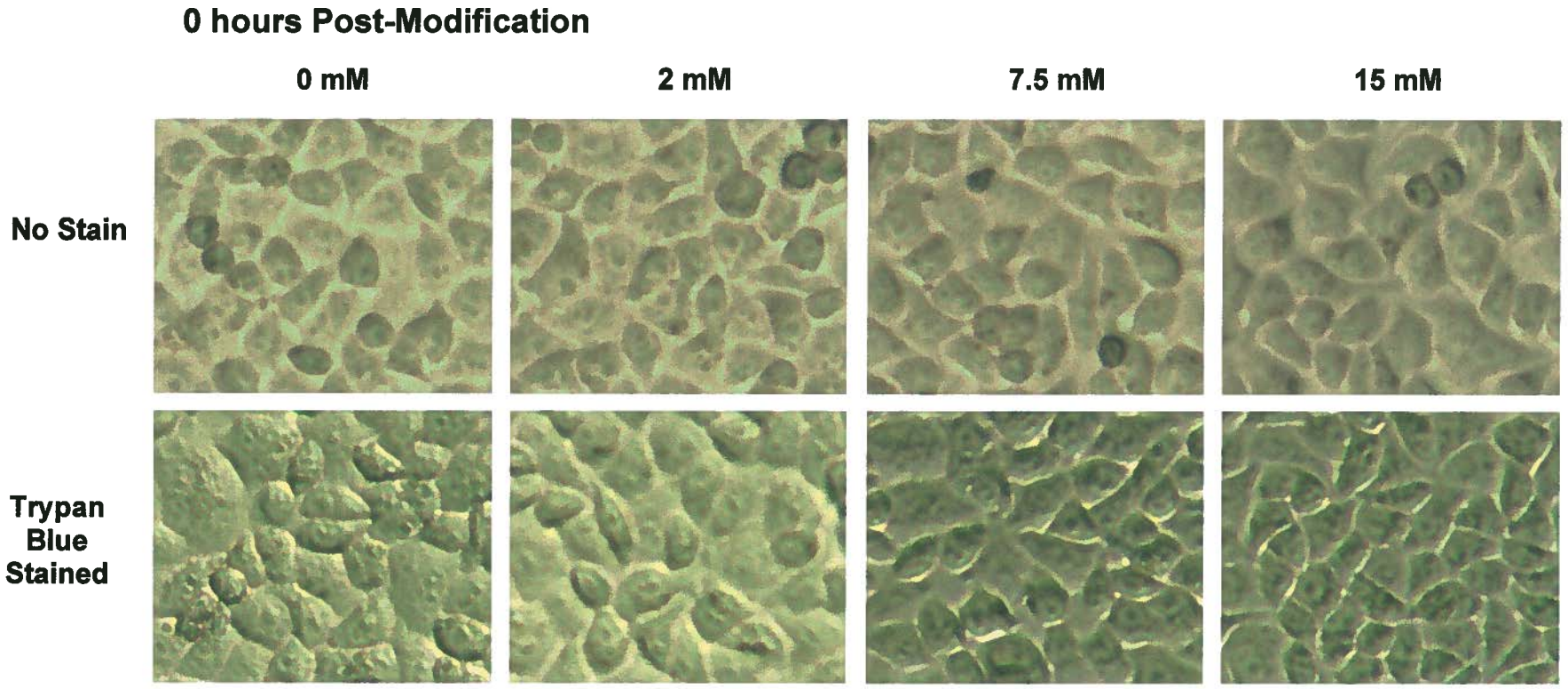


Figure 5.7 Images of Unstained and Trypan Blue Stained HeLa Cells Modified with 20 kDa SVAmPEG at 0 Hours Post-modification. Cells were modified under standard conditions. Representative images of two experiments performed in quadruplicate.

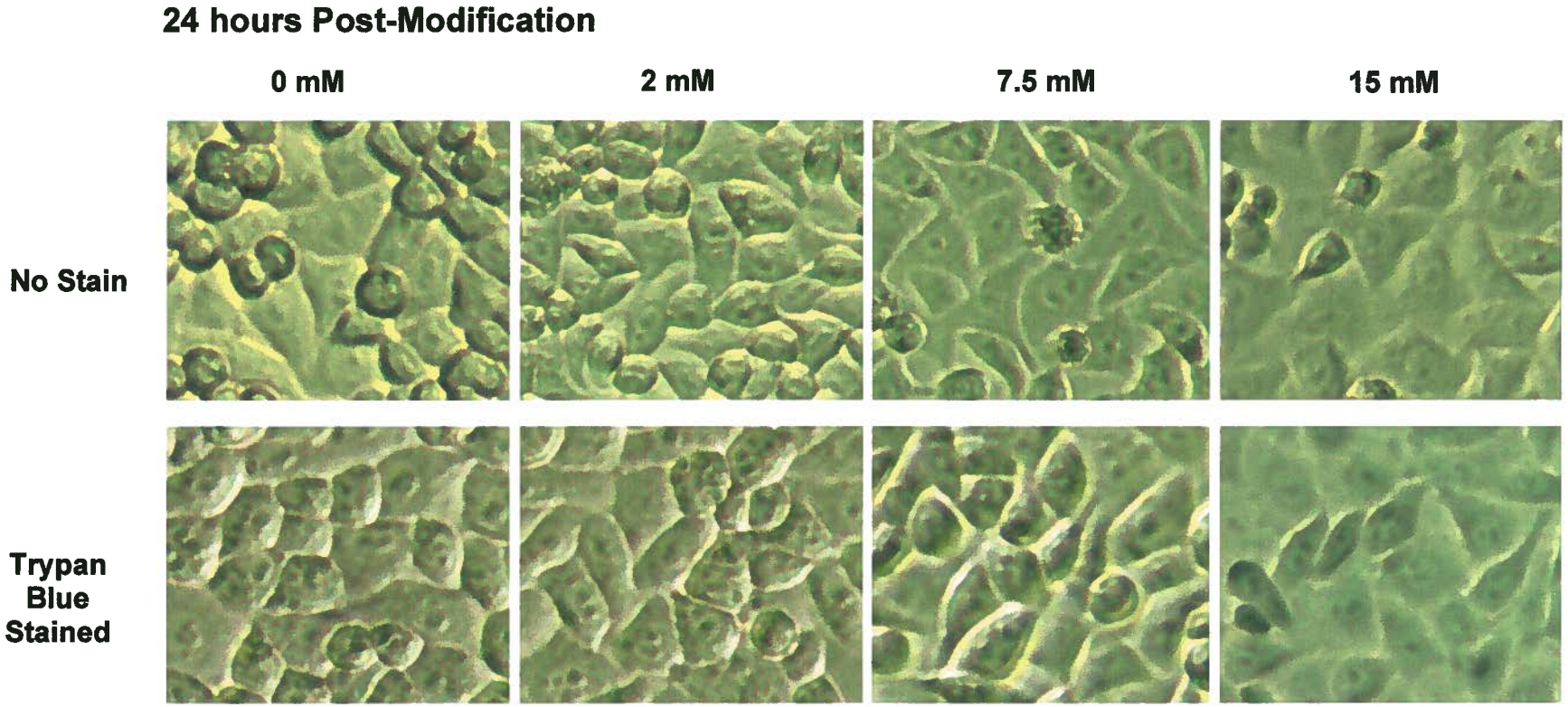


Figure 5.8 Images of Unstained and Trypan Blue Stained HeLa Cells Modified with 20 kDa SVAmPEG at 24 Hours Post-modification. Cells were modified under standard conditions. Representative images of two experiments performed in quadruplicate.

Table 5.2. mPEG-Modification of HeLa Cells Does Not Dramatically Decrease Cell Viability.

	Number of Dead Cells Per Well			Estimated Percent Viability		
Condition	**0 hours**	**24 hours**	**48 hours**	**0 hours**	**24 hours**	**48 hours**
Negative Control	64 ± 9	308 ± 35	582 ± 16	99.95 ± 0.01	99.88 ± 0.01	99.86 ± 0.01
0 mM	79 ± 13	295 ± 19	559 ± 21	99.94 ± 0.01	99.88 ± 0.01	99.87 ± 0.01
2 mM	61 ± 7	286 ± 22	561 ± 24	99.95 ± 0.01	99.88 ± 0.01	99.88 ± 0.01
7.5 mM	* 120 ± 10	322 ± 13	623 ± 19	* 99.91 ± 0.01	99.87 ± 0.01	99.85 ± 0.01
15 mM	* 147 ± 10	* 449 ± 51	646 ± 27	* 99.89 ± 0.01	* 99.82 ± 0.02	99.85 ± 0.01

Estimated percent viability based on the number of cells in the 0 mM condition. The average number of cells per well were as follows: 0 hours = 133,350 cells/well, 24 hours = 246,600 cells/well, and 48 hours = 423,000 cells/well.
Values expressed are the mean ± SE of two separate experiments performed in quadruplicate.
* indicates significantly different from the negative control, 0 mM, and 2 mM conditions ($p<0.023$)

5.5 SUMMARY OF mPEG-MODIFICATION OF HOST CELLS

In initial experiments using fluorescein-labeled mPEG, we found that under our grafting conditions mPEG binds to the cell surface proteins with an even distribution. Subsequently, we modified non-polarized cells with 2 or 20 kDa mPEG and challenged them with a broad range of virus doses. At all virus doses, grafting of 20 kDa mPEG resulted in a dramatic dose-dependent decrease in infection. Specifically, at grafting concentrations of 7.5 mM we observed a dramatic decrease in infection (*i.e.* MOI of 0.1, >65% reduction at all times), and at grafting concentrations of 12.5 and 15 mM infection was almost completely prevented. In contrast, grafting of 2 kDa mPEG to the host cells provided no protection against infection. Therefore, in the context of host cell modification, larger polymers provide enhanced protection.

As RSV infects polarized cells *in vivo*, we examined host cell modification with polarized MDCK cells. In this cell line, we found that mPEG-modification resulted in a reduction of infection by more than 80% at both 24 and 48 hours post challenge. Moreover, mPEG-modification also prevented the subsequent spread of infection by progeny virus. Lastly, we examined cell viability and found that dislodging the PEGylated cells from the tissue culture plate resulted in some cellular toxicity. This was indicated by the observation that with PI staining there was no enhanced toxicity over time. Importantly, performing in-plate trypan blue staining mitigated these effects, and we found that at all mPEG grafting concentrations cells had a greater than 99.8% viability. Taken together, our findings show that mPEG-modification of the host cells is a highly effective strategy for preventing RSV infection and indicate that this strategy will be protective *in vivo*.

CHAPTER 6: DISCUSSION

6.0 OVERVIEW

For purposes of clarity, the discussion is divided into four sections. In the first section, we discuss mPEG-modification of RSV, and in the second, we discuss mPEG-modification of host cells. In the last two sections, we compare the two strategies, and then draw conclusions and discuss the overall impact our findings. Importantly, a summary of the effects of 2 and 20 kDa mPEG grafting to GFP-RSV or the host cells (HeLa) over log scale virus doses are given in Tables 4.4 and 5.1, respectively. As demonstrated by these tables, both PEGylation strategies result in significant protection against RSV infection and propagation. However, there were important and contrasting effects with different polymer lengths in the two strategies.

6.1 mPEG-MODIFICATION OF RSV

6.1.1 Linker Chemistry and Polymer Length in mPEG-Mediated Inhibition of RSV Infection

Based on studies with mPEG-modified SV40 (116), we hypothesized that covalent grafting of mPEG directly to RSV would prevent infection. In support of our hypothesis, we found that mPEG-modification of RSV prevented infection in a dose-dependent manner. This was demonstrated by plaque assay experiments and over all virus doses in the log scale studies. We further showed by plaque assay, that in comparison to all other linker chemistries, the SVA linker conferred a greater inhibition of infection.

The superiority of the SVA linker chemistry is most readily explained by examining the hydrolysis half-lives of the different linker chemistries (see Introduction Table 1.1). SCmPEG and CmPEG have hydrolysis half-lives of 20.4 and ~10 minutes, respectively, while SVAmPEG

has a hydrolysis half-life of 33.6 minutes (119, 120). Thus in aqueous solution, SVAmPEG will undergo hydrolysis at a much slower rate than SCmPEG and CmPEG. Consequently, more mPEG will remain activated over the 30 minute incubation period resulting in enhanced grafting and significantly greater inhibition of the virus.

Subsequent to these studies, we evaluated the effect of polymer length on RSV inactivation. Initially, we performed plaque assays with both the SC and SVA linker chemistries. In these experiments, we compared 2 and 5 kDa SCmPEG, and found that the shorter 2 kDa species demonstrated improved protection. With the SVA linker chemistry, we evaluated polymer lengths of 2, 5, and 20 kDa, and found that 2 and 5 kDa provided greater protection than 20 kDa at only the 5 mM grafting concentration. However, in subsequent log scale GFP-RSV experiments, we found that relative to 20 kDa SVAmPEG, the 2 kDa polymer consistently provided enhanced protection at low to moderate (2-7.5 mM) grafting concentrations. Not surprisingly, at high grafting concentrations (12.5 and 15 mM) both 2 and 20 kDa mPEG provided similar levels of protection, and almost completely abolished RSV infection. These effects with high grafting concentrations are likely a result of enhanced polymer grafting to the virion.

To understand the effects of polymer length, we must examine the distribution of viral proteins on the surface of RSV. On the RSV virion, the G and F proteins, which mediate attachment and fusion, respectively, form glycoprotein "spikes" (4). These "spikes" are 6 to 10 nm apart and extend 11-20 nm from the virion surface. Looking at the physical lengths of the 2 and 20 kDa polymers (see Introduction, Figure 1.11), we see that 2 kDa mPEG has a length and therefore, radius of gyration, of ~ 6 nm, while 20 kDa mPEG has a length of ~50 nm. Importantly, these lengths represent the average radius of gyration (R_g), and both polymers have a maximum radius of gyration ($R_g$max) that also exerts steric hinderance. Thus, as illustrated in Figure 6.1, the 2 kDa polymer will graft to individual glycoprotein "spikes", and the $R_g$max will

not easily interfere or inhibit grafting of other small polymers to adjacent "spikes". This will result in the majority of G and F proteins being modified even at low grafting concentrations. In contrast, at low to moderate grafting concentrations, the $R_g$max of grafted 20 kDa polymers will block or limit access of mPEG to spikes over a much larger area. Therefore, more of the G and F proteins will remain unmodified. This may in turn, allow the surface proteins to interact with the host cell receptor leading to viral invasion. To test this hypothesis, future experiments could be performed comparing RSV-modified with radiolabeled 2 or 20 kDa mPEG.

At high grafting concentrations, it is likely that the steric inhibition of grafted 20 kDa polymers, which prevents grafting to adjacent spikes will be at least partially overcome. Thus, regardless of polymer length, at high concentrations both 2 and 20 kDa mPEG will likely graft at a high density. This will result in the formation of a dense mPEG layer or brush border on the surface of the virion and will effectively inhibit infection.

Importantly, our findings of differing efficacy with short and long mPEG polymers are in contrast to those on mPEG-modified SV40 (135). Previously, McCoy *et al.* evaluated modification of SV40 with mPEG ranging in length from 2 to 20 kDa, and at grafting concentrations from 0 to 15 mM. At low to moderate grafting concentrations, they found that polymer length did not alter the level of viral inactivation. However, at high grafting concentrations they found, as we did, that polymer length had no significant effect. As explained below, this discrepancy between our findings and those of McCoy *et al.* at low to moderate grafting concentrations, is likely the result of differences in the surface distribution of proteins and, therefore, lysine residues on SV40 and RSV.

SV40 is a non-enveloped virus with an icosahedral capsid (172). This capsid is composed of 72 viral protein 1 (VP1) pentamers in a highly ordered conformation. Importantly, each VP1 pentamer contains 25 lysine residues. Therefore, on the SV40 surface, lysine residues are evenly spaced and distributed. In contrast, the RSV virion has a bilipid envelope obtained

during maturation and budding from its host cell. This envelope contains both host cell and viral proteins. Given the nature of host cell surface proteins, within the RSV envelope there will be sufficient variation in host protein size and distribution. Furthermore, focusing on the viral proteins, these proteins vary in their distance from each other (6-10 nm) and their height from the virion surface (11-20 nm) (4). As well, the proteins are also capable of movement within the lipid bilayer (162). As a result, when subject to PEGylation at low to moderate grafting concentrations, SV40 with its highly regular distribution of lysine residues will likely have a very consistent distribution of mPEG polymers grafted to its surface. This will likely mitigate any effects of polymer length.

In contrast, given RSV's more varied surface topography and the large radius of steric hindrance generated by 20 kDa polymers, grafting of low to moderate concentrations will likely result in a more patchy distribution of mPEG. In turn, significantly more of the virion surface will remain unmodified, therefore, resulting in a higher degree of infectivity. Similar to SV40 modification, 2 kDa mPEG will likely graft at a much higher density with a more even distribution as a result of its small size. This will likely overcome variations in viral surface topography and more effectively prevent infection. Given that with both SV40 and RSV, high grafting concentrations of both short and long polymers inhibit infection, it is likely that at these concentrations mPEG chains pack closely together and form dense exclusion zones. The formation of these dense exclusion zones likely overcomes variations in viral surface topography, thus resulting in similar levels of viral inhibition.

Considering higher virus doses as in the log scale studies, we found that even at high MOI, grafting of ≥7.5 mM, 2 kDa SVAmPEG usually resulted in near complete inhibition. Therefore, it is likely that at this 7.5 mM grafting concentration, mPEG is grafting at levels that hide most of the viral surface proteins. At lower MOI, we see that lower grafting concentrations

produce similar levels of inhibition. This is likely the result of relatively fewer targets for mPEG, thus resulting in a much higher mPEG:target ratio (*i.e.* relative grafting concentration).

As the efficacy of mPEG-modification of RSV in non-polarized HeLa cells was encouraging, we further sought to evaluate mPEG-modification of RSV in a more biologically relevant cell line. As RSV infects polarized cells *in vivo*, we evaluated mPEG-modification of RSV with the polarized MDCK cell line. Thus, we modified GFP-RSV with 7.5 mM, 2 kDa SVAmPEG and challenged these cells. In agreement with our findings in non-polarized cells, we found that mPEG modification of RSV dramatically inhibited infection. Importantly, this suggests that mPEG-modification inhibits infection by disrupting similar mechanisms of virus-host cell receptor interactions in both cell types, and indicates that mPEG-modification of RSV will likely prevent infection *in vivo*.

A potential concern for these studies was that mPEG-grafting to the virus might disrupt the virion. To ensure that this was not the case, we performed TEM studies on mPEG-modified RSV. We showed that the GFP-RSV virion remain intact over mPEG grafting concentrations from 0-15 mM. Our findings are consistent with similar EM studies on mPEG-modification of adenovirus, which also showed that mPEG-modification did not dramatically disrupt the integrity of the virion (168).

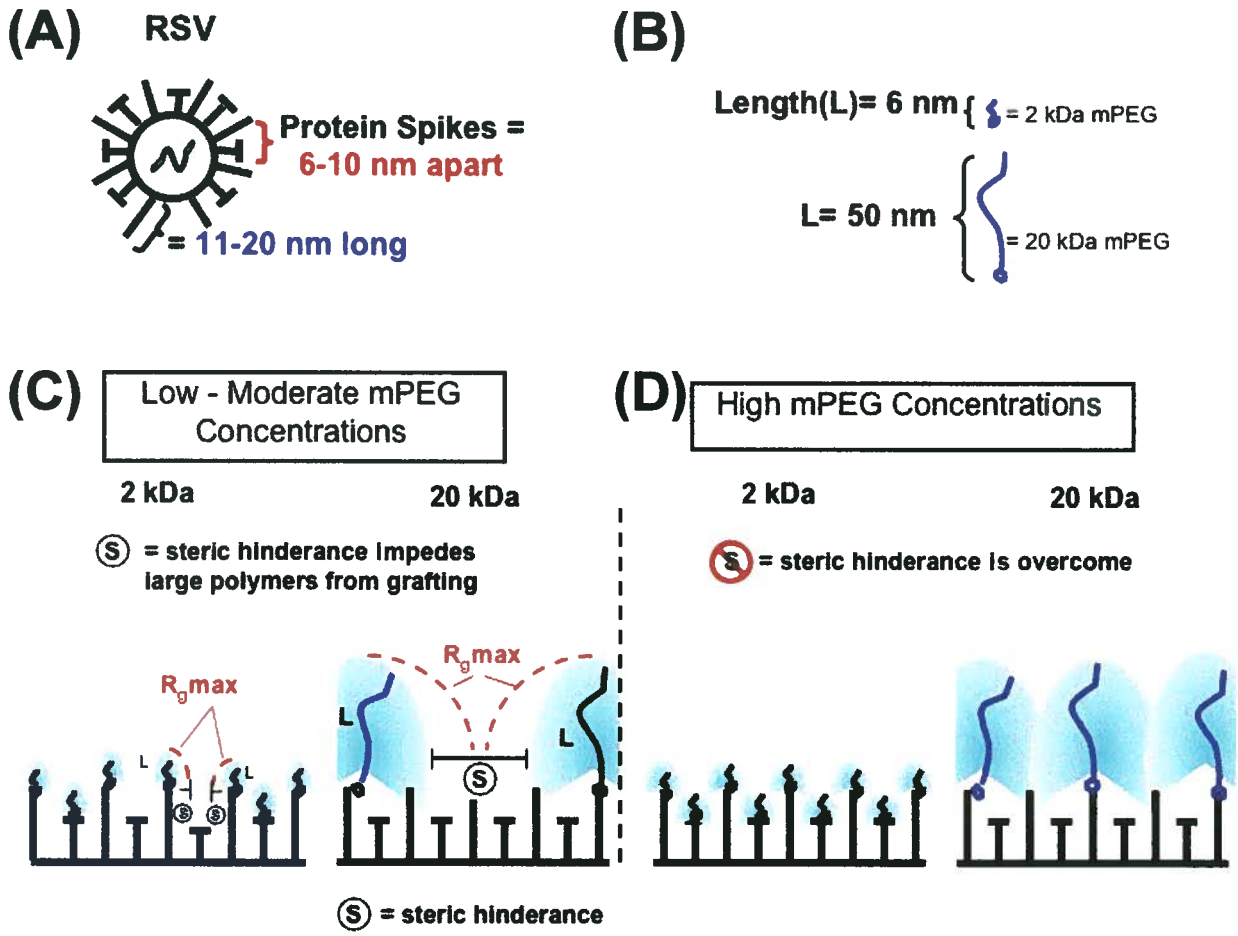


Figure 6.1. Distant Between G and F Protein "Spikes" on the RSV Virion Surface Explains Differing Degrees of Protection Conferred by Grafting of 2 and 20 kDa mPEG at Low to Moderate Concentrations. Note: Grafted mPEG will on average assume a mushroom conformation illustrated in blue. However, the mPEG will also have a maximum radius of gyration (R_g max) that will at times extend beyond the high-density mushroom shape and exert steric effects. Illustrated in **(A)** is a schematic of RSV showing the viral surface proteins which form "spikes" 11-20 nm long and 6-10 nm apart. In **(B)** the length of the 2 and 20 kDa polymers are illustrated. Shown in **(C)** and **(D)** are magnified schematics of the viral surface at high and low mPEG grafting concentrations. From **(B)**, 2 kDa mPEG has a radius of gyration of ~6 nm, and therefore as shown in **(C)** at low to moderate concentrations would readily graft to almost every "spike" and exert little steric hinderance on the grafting of short polymers to adjacent proteins. In contrast, 20 kDa mPEG has a radius of gyration of ~50 nm, thus on the virus surface only a few "spikes" would be modified and the grafted polymer would sterically hinder other large polymers from grafting. As the polymer rotated this would potentially expose unmodified G and F proteins allowing the virus to infect a host cell. This may account for the differences in protection conferred by 2 and 20 kDa mPEG at low to moderate grafting concentrations. **(D)** At high grafting concentrations, steric hinderance will be obercome and both 2 and 20 kDa will graft at high density to RSV and inhibit infection. Relative polymer sizes are drawn to scale to the viral proteins.

6.1.2 Inhibition of RSV Infection by mPEG Grafting to the G or F proteins.

Review of the literature shows that other groups have previously used polymers to inhibit RSV infection. Specifically, Hosoya *et al.* (1991) showed that incubation of RSV with sulfated polysaccharides or sulfated polyvinylalcohol prevented RSV infection (180). An important difference between mPEG-modification and the use of these polymers is that mPEG-modification results in the formation of a covalent and relatively permanent bond to the virus. Furthermore, incubation of SV40 with soluble (unbound) mPEG has minimal effect on infection rates (116). In contrast, the polymers evaluated by Hosoya *et al.* bind to RSV non-covalently, and therefore, can dissociate from the virus. Through their work, Hosoya *et al.* found that the sulfated polysaccharides and polyvinylalcohols prevented infection of host cells by several viruses including influenza A, parainfluenza 3, human immunodeficiency virus (HIV), and RSV (180). As these polymers prevented both binding and fusion of most of the viruses studied, the authors compared the F-protein sequences of these viruses. Within the various F proteins, they found a conserved amino acid sequence of phenylalanine-leucine-glycine-phenylalanine-leucine. Consequently, the authors hypothesized that this region was important or crucial in the interactions between the viral F proteins and the host cell receptors (180). More recent studies have shown that this region is responsible for fusion of the viral envelope with the host cell membrane and is part of the fusion peptide with the mature F protein (181, 182).

As shown in Figure 6.2(a), the RSV F protein is initially translated as the precursor F_o protein. This protein is subsequently cleaved by furin at amino acid residues 109 and 136 to release a 27 amino acid peptide designated pep27 (181, 183, 184). The remaining two peptides consisting of amino acids 1-108 and 137-154 are designated F_2 and F_1, respectively (184). The F_1 peptide forms the major structure of the F protein (see Figure 6.2(b)) and the F_2 peptide is bound parallel to F_1 by two disulfide bonds (183, 185). On the viral surface, RSV F proteins form trimers and the fusion peptide is concealed within the three-dimensional structure of the

trimeric protein (see Figure 6.2(b)) (182). Upon binding to the host cell surface, the F protein trimer undergoes a conformation change and inserts the fusion peptide into the host cell membrane. Subsequently, the F protein undergoes further conformational changes leading to fusion of the viral and host cell membrane (182, 186).

Importantly, while the fusion peptide is hidden within the structure of the F protein, as shown in Figure 6.2(a), the mature F protein is rich in lysine residues (185) rendering it susceptible to PEGylation. Therefore, it is likely that mPEG will graft to the RSV F protein and may sterically prevent the F protein from interacting with its host cell receptor. Previous studies on mPEG-modified RBC have shown that mPEG-grafting prevents the focal clustering of complement receptor 1 (CR1) and the subsequent formation of immune complexes on the RBC surface (162). Therefore, mPEG grafting could also disrupt the trimeric structure of the F protein or prevent the focal clustering of several F protein trimers required to induce fusion.

In addition to interacting with the RSV receptor, RSV also interacts with heparan sulfate (HS) on the cell surface (83, 84). HS is thought to be a co-receptor for RSV, as its digestion from the cell surface decreases, but does not completely abrogate infection (81). Importantly, both the G and F proteins bind HS via electrostatic interactions between positively charged amino acids in their heparan- binding domains (HBD) and the negatively charged sulfate groups of HS. As mPEG-modification has previously been shown to camouflage the surface charge and inhibit interactions of RBCs (111, 145, 161, 163), grafting of mPEG to RSV will similarly prevent the interaction between HS and RSV by both decreasing the zeta potential (*i.e.* hiding the positively charged amino acids) of the HBD and by sterically hindering interactions with HS (see Figure 6.3). As a result, it is likely that mPEG modification of the G and F proteins will completely inhibit RSV from interacting with both its cell surface receptor and co-receptor.

(A)

NH_2-Terminal End

1 MELLILKANA ITTILTAVTF CFASGQNITE EFYQSTCSAV SKGYLSALRT GWYTSVITIE

61 LSNIKENKCN GTDAKVKLIK QELDKYKNAV TELQLLMQST PPTNNRARRE LPRFMNYTLN

121 NAKKTNVTLS KKRKRRFLGF LLGVGSAIAS GVAVSKVLHL EGEVNKIKSA LLSTNKAVVS

181 LSNGVSVLTS KVLDLKNYID KQLLPIVNKQ SCSISNIETV IEFQQKNNRL LEITREFSVN

241 AGVTTPVSTY MLTNSELLSL INDMPITNDQ KKLMSNNVQI VRQQSYSIMS IIKEEVLAYV

301 VQLPLYGVID TPCWKLHTSP LCTTNTKEGS NICLTRTDRG WYCDNAGSVS FFPQAETCKV

361 QSNRVFCDTM NSLTLPSEIN LCNVDIFNPK YDCKIMTSKT DVSSSVITSL GAIVSCYGKT

421 KCTASNKNRG IIKTFSNGCD YVSNKGMDTV SVGNTLYYVN KQEGKSLYVK GEPIINFYDP

481 LVFPSDEFDA SISQVNEKIN QSLAFIRKSD ELLHNVNAGK STTNIMITTI IIVIIVILLS

541 LIAVGLLLYC KARSTPVTLS KDQLSGINNI AFSN-COOH Tail

K = lysine residue
FLGFL = fusion peptide
Red Font and Underline = pep27 amino acid sequence that is cleaved from the RSV F_0 protein
Amino acid residues 1-108 = F_2 protein. Amino acids 137-574 = F_1 protein.
No Underline = protein sequence on outer virus surface
Black Underline = protein sequence embedded in the membrane

(B)

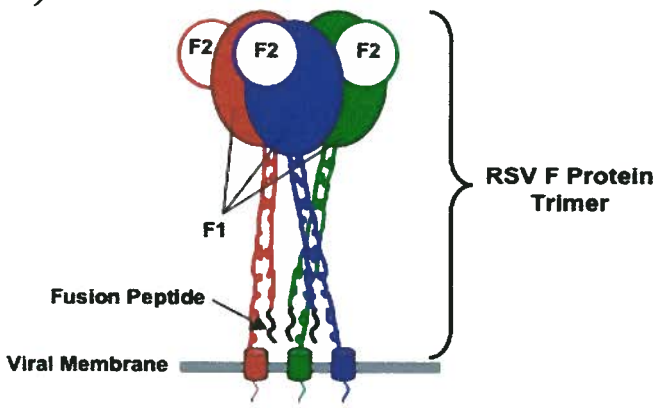


Figure 6.2. RSV Fusion Protein Sequence and Schematic. The RSV F protein has previously been shown require the Phenylalanine-Leucine-Glycine-Phenylalanine-Leucine (FLGFL) domain illustrated above for fusion with the host cell membrane (171, 172). Importantly, there are several Lysine (K) residues in the F protein that could be PEGylated, and, therefore, prevent RSV infection. The F protein is cleaved to remove a 27 amino acid sequence known as p27. The remaining two lengths of the peptide are designated F_1 (amino acids 137-574) and F_2 (amino acids 1-108) which are then linked in parallel by disulphide bonds to form the mature RSV F protein (180-185). **(B)** Schematic Showing the Structure of Mature RSV F protein. The RSV F protein exists as a trimer on the viral surface. As illustrated above the fusion peptide is hidden within the 3 dimensional structure of the protein near the viral surface. Upon binding of the G protein to the host cell, the F protein undergoes a change in conformation and the fusion peptide is pushed forward and inserted into the host cell membrane (182, 186).

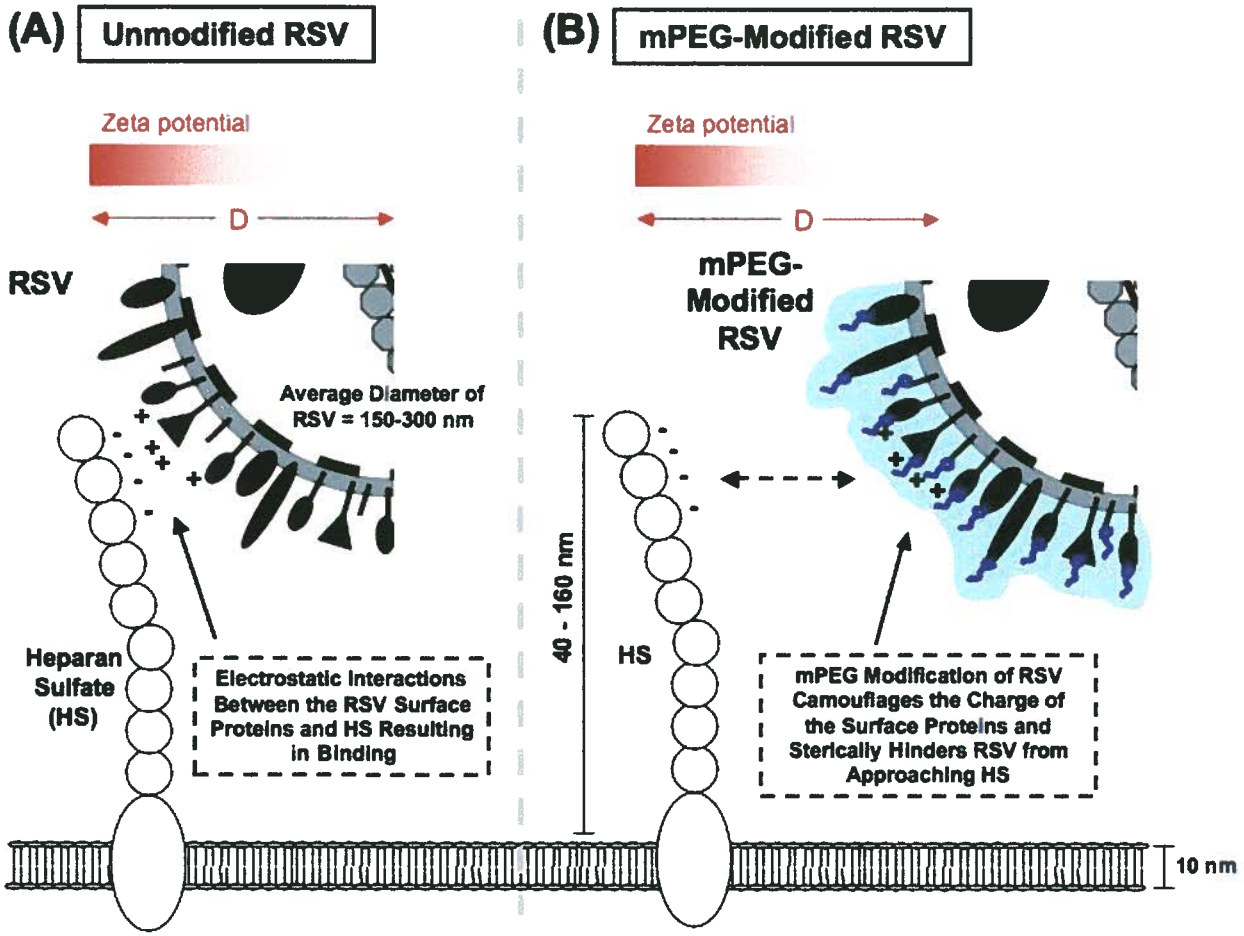


Figure 6.3. mPEG-Modification of RSV Camouflages the Charge of the Viral Surface Proteins and Sterically Inhibits the Virus from Interacting with Heparan Sulfate (HS). (A) RSV normally interacts with HS via interactions between positively charged regions in the G and F proteins, and negatively charged sulfate groups on HS. **(B)** mPEG-modification of RSV camouflages the positively charged regions on the viral surface proteins and sterically inhibits RSV from interacting with HS. Drawn to approximate scale.

6.1.3 Clinical Applications for mPEG-Modification of Viruses

Considering the clinical uses for mPEG-modification of viruses, there are two potential applications. The first is as a pathogen reduction system in blood products. Importantly, we are the first group to report mPEG-modification and inactivation of an enveloped virus. As viral pathogens in blood products are both enveloped and non-enveloped, our findings support the use of mPEG-treatment of blood products as a broad-spectrum anti-viral pathogen reduction strategy. This approach would be most applicable in blood systems of underdeveloped nations in which nucleic acid testing is cost prohibitive. Though not investigated in this thesis, mPEG-modification of blood products could inactivate several viruses such as CMV, Hepatitis A, B, and C, and HIV, and as a result, dramatically reduce the transmission of blood borne infections in these nations.

The second potential use of mPEG-modified viruses is as a vaccine strategy. Previously, Mizouni *et al.* (2000), have shown that administration of mPEG-modified SV40 to mice enhanced the antibody response against the SV40 surface proteins (171, 178). Importantly, recent studies on RSV vaccines have shown that with repeat administration of previously ineffective vaccines mice developed a protective high affinity antibody response (187). Given that mPEG-modification of proteins and xenogenic RBC has been shown to prolong circulation half-life (109, 111), administration of mPEG-modified RSV would result in prolonged and repeated exposure to viral antigens, and could similarly induce this protective antibody response. This may in turn lead to the development of the first safe and effective RSV vaccine, and may also lead to the development of a novel vaccine strategy (188).

6.1.4 Future Studies on mPEG-Modified Virus

In summary, mPEG-modification of RSV prevented viral infection confirming our hypothesis. We further hypothesize that polymer linker chemistry and length specific effects can be attributed to hydrolysis half-life and viral surface protein distribution or topography. Our findings are particularly exciting, as they support the use of mPEG-modification as a pathogen reduction system in blood products and as a potential vaccine strategy.

In future studies, the *in vivo* efficacy of both these applications should be evaluated as they may offer novel means of viral inactivation and prophylaxis. Unfortunately, current animal models of RSV may not be fully suitable for this purpose. Other potential animal models for evaluating PEGylated virus as a vaccine include Sendai virus (SeV) or mouse pneumovirus (PVM), as both of these virus strains are capable of causing pronounced disease and illness in mice (189, 190). In the context of blood borne viruses, the use of mouse hepatitis virus (strains 2 or 3) would permit evaluation of intravenous viral transmission and, therefore, allow assessment of mPEG-mediated viral inactivation of blood products (191).

6.2 mPEG-MODIFICATION OF HOST CELLS

6.2.1 Effect of Polymer Length on mPEG-Mediated Host Cell Protection

The camouflage of host cells is a more relevant approach to preventing RSV (and other respiratory virus) infection as mPEG could be easily formulated in a nasal spray. In initial experiments, we demonstrated that mPEG grafts to the host cell surface using fluorescein-labeled mPEG. However, it appeared that grafting of the 5 kDa fluorescein-SVAmPEG had some cellular toxicity in HeLa cells. Importantly, this was not observed with 2 or 20 kDa SVAmPEG shown both microscopically and by viability staining. In addition, previous work by McCoy *et al.,* (116, 135) reported no cellular toxicity for several host cell lines modified with all polymer lengths examined including 2, 3.4, 5, and 20 kDa mPEG (116, 135). This suggests that the fluorescein or a contaminant in the fluorescein-mPEG may have been toxic to the cells. Alternatively, for the HeLa cell line, the 5 kDa mPEG polymer may be of an appropriate length and grafting at a density capable of interfering with the binding of growth hormones and signaling molecules in the media. Relative to the 5 kDa polymer, the 2 kDa mPEG species while similarly grafting at high density may be too short to disrupt cell signaling, and the longer 20 kDa polymers may graft at a much lower density and not affect cell signaling.

In subsequent experiments, we proved our hypothesis that mPEG-modification of the host cell would prevent or inhibit RSV infection. As demonstrated in Chapter 5, we found that at all MOI evaluated, grafting of 20 kDa mPEG to the host cell inhibited RSV infection in a dose-dependant manner. Indeed, at grafting concentrations of $\geq$ 7.5 mM, viral invasion and propagation was inhibited even at very high MOI. At MOI more relevant to natural inoculation doses (*i.e.* MOI 0.01), a grafting concentration of $\geq$ 7.5 mM virtually abolished viral infection.

In contrast, grafting of 2 kDa mPEG to the host cell did not confer any significant protection. This finding was surprising as previous studies with mPEG-modified host cells for SV40, rat coronavirus (RCV), mouse adenovirus (MAV), Theiler's murine encephalomyelitis

(TMEV), and cytomegalovirus (CMV) demonstrated some efficacy with small polymers though large polymers were superior (135). These differences between our findings and those of previous studies are most likely explained by theorizing about interactions of RSV with its unknown host cell receptor.

First, considering the variation in protection seen with the 2 and 20 kDa mPEG polymers, our findings may provide some evidence about the size of the RSV receptor. As illustrated in Figure 6.4 (a,b,c), when 2 kDa mPEG is grafted to the cell surface it will produce an mPEG zone of exclusion that extends approximately 6 nm above the surface. In contrast, 20 kDa mPEG will project approximately 50 nm from the surface, therefore, creating a much deeper mPEG zone of exclusion. Given the differing efficacies of the two polymer lengths, it is possible that the RSV receptor extends more than 6 nm above the cell surface, but does not extend past 50 nm. Thus, the RSV receptor would be effectively hidden by the long 20 kDa polymer, but remain unprotected by the short 2kDa polymer.

Alternatively, given that host cells previously evaluated likely have viral receptors that extend more than 6 nm from the cell surface, the discrepancies between our findings and those in other cells may be explained by examining the host cell receptors. Currently, the high affinity receptor that induces fusion of the RSV envelope with the host cell membrane is unknown. The receptor may be a heavily glycosylated protein or a carbohydrate rendering it relatively unsusceptible to mPEG modification. Another hypothesis, as shown in Figure 6.4 (a,b,c), is that the receptor may be surrounded by large heparan sulfate molecules that are not readily modified by mPEG. Thus, while both the 2 and 20 kDa polymers could not bind directly to the receptor, they could graft to proteins in close proximity. Grafting of 2 kDa mPEG would not extend far enough above the cell surface to prevent interactions of the virus with its receptor. However, a 20 kDa polymer in close proximity to the receptor could physically exclude RSV and also obscure electrostatic interactions between the viral surface proteins and the host cell receptor.

Another possibility is that the RSV receptor may extend beyond the ~50 nm zone of exclusion created, however, as shown in Figure 6.4(d), upon binding to RSV, the receptor may not be able to pull the virus into close proximity to the cell surface.

Focusing our attention on previous studies of mPEG-modified host cells, McCoy *et al.* showed that mPEG-modification of the host cell surface with long chain polymers provided enhanced protection compared to short chain polymers. However, even with short chain polymers, McCoy *et al.* showed some protection against infection. This is likely a result of differences between the RSV receptor and the receptors for the other viruses examined. Importantly, of the known receptors for the viruses examined by McCoy *et al.* all of them were relatively small (see Table 1.2) (135). Therefore, both short and long chain polymers could camouflage these receptors, and confer protection regardless of the polymer length. In contrast, as suggested by our findings, the RSV receptor could be a large molecule extending more than 6 nm from the surface, and would therefore, require a thick mPEG zone of long chain polymers to exclude the virus. Importantly, as shown in Figure 6.4, grafting of different polymer lengths to the host cell surface provided insight into the size of the RSV receptor, our work provides a novel method for characterizing unknown virus receptors.

Subsequent to evaluating the efficacy of mPEG-modification of host cells against RSV infection, we assessed if mPEG-grafting resulted in a decrease in cell viability. We found that by both PI and trypan blue staining, moderate to high grafting concentrations (7.5 and 15 mM) of mPEG decreased cell viability. However, much of this toxicity was associated with dislodging the cells from the plate. By PI staining viability was ~50-65% with 7.5 and 15 mM mPEG grafting, respectively; however, by in-plate trypan blue staining viability increased to 99.8% with both 7.5 and 15 mM mPEG grafting. Importantly, while viability by trypan blue staining was still significantly decreased from 99.9% (0 mM) to 99.8% with mPEG grafting, this decrease was not biologically significant. Furthermore, this small decrease in viability does not account for

the dramatic protection conferred by mPEG grafting, and by 48 hours post-modification there were no differences in cell viability. In addition, experiments performed by McCoy *et al.* on host cell PEGylation similarly reported little or no associated cell toxicity (116, 135). Thus, taken together, our findings show that mPEG-modification is safe and non-toxic (192), and is capable of forming a potent barrier to viral invasion and propagation.

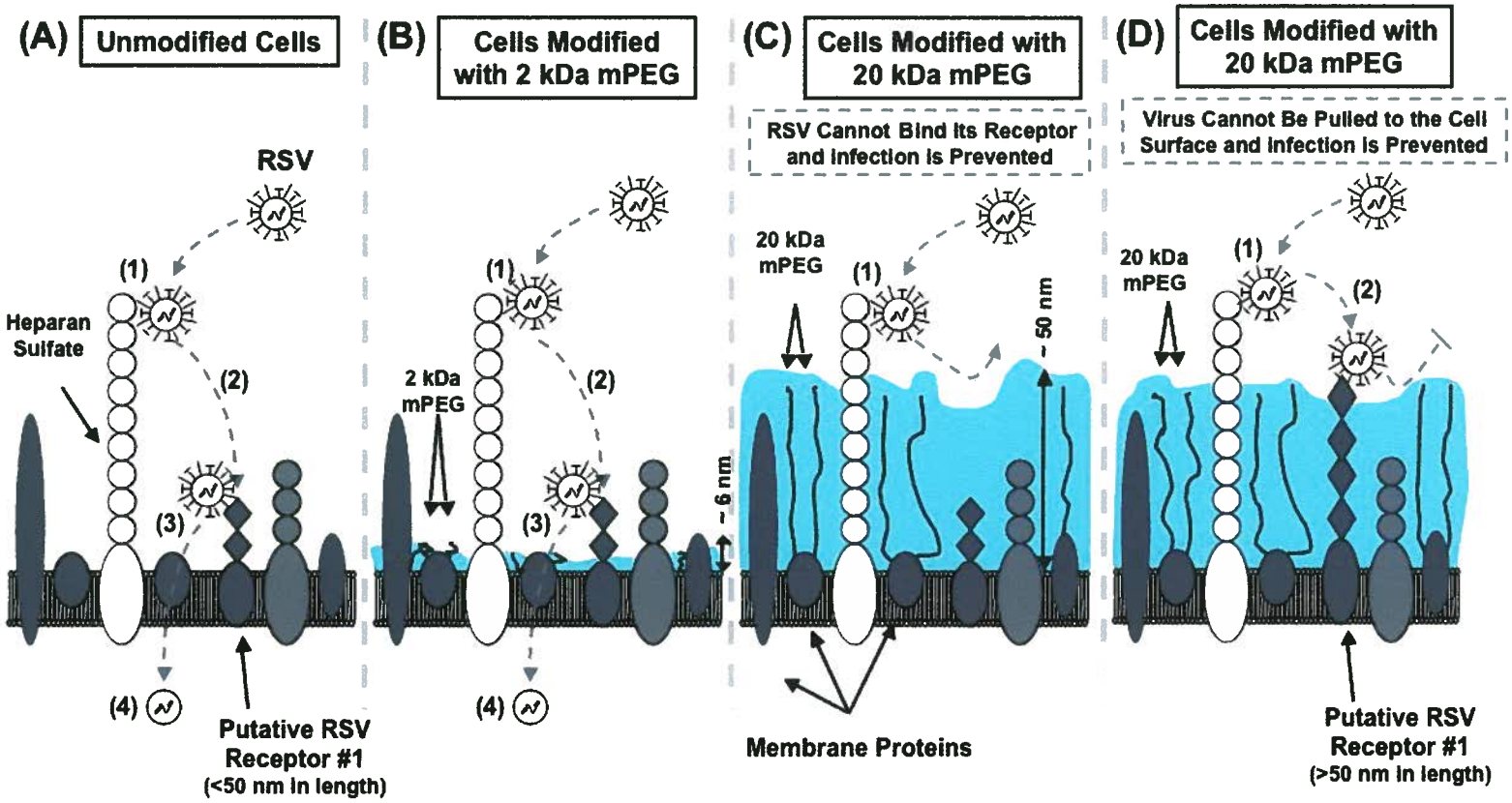


Figure 6.4. mPEG-Modification of the Host Cell with 20 kDa mPEG Likely Inhibits Heparan Sulfate (HS) from Helping RSV Find Its Cell Surface Receptor or Prevents RSV Bound to Its Receptor from Approaching the Cell Surface. However, grafting of 2 kDa mPEG Does Not Prevent These Interactions. **(A)** RSV infection of an unmodified host cell. RSV binds HS (1) and (2) HS facilitates RSV in finding its cell surface receptor, (3) RSV binding to its unknown receptor, (4) RSV is pulled into close proximity with the cell surface and fuses with the cell membrane resulting in infection. **(B)** Grafting of 2 kDa mPEG to the host cell produces an mPEG zone of exclusion that does not hide the RSV receptor or prevent RSV from being pulled into close proximity to the cell surface. Therefore, (1) HS is able to bind and (2) help RSV bind its receptor (3) resulting in (4) viral infection. **(C)** Grafting of 20 kDa mPEG produces an mPEG zone of exclusion that (1) does not prevent HS from binding RSV, but does not permit the HS-RSV complex to approach the virus receptor and infection is prevented. **(D)** Alternatively, the putative receptor could extend above the ~50 nm zone of exclusion. RSV could still binds HS (1), and HS would facilitate RSV in finding its receptor (2), however, due to thick mPEG layer, the virus could not be pulled through the zone of exclusion and infection is prevented.

6.2.2 Clinical Applications of mPEG-Modification of Host Cells

Respiratory viruses including RSV, rhinoviruses, coronaviruses, and adenoviruses are a major cause of human morbidity and mortality. Based on our results with RSV, the most obvious clinical application of mPEG-modification of the host cells would be as a prophylactic anti-viral nasal spray. Given that RSV infects polarized cells *in vivo*, our results showing that 20 kDa mPEG grafting inhibited RSV infection of polarized MDCK cells, further supports the use of mPEG as a nasal spray. Across most virus doses examined, an mPEG grafting concentration of 7.5 mM provided a high level of protection. Thus, this grafting concentration should be evaluated *in vivo*. Furthermore, considering the viscosity of concentrated 20 kDa mPEG solutions, 7.5 mM has a relatively low viscosity compared to mPEG solutions of 12.5 and 15 mM. Therefore, in a nasal spray, this concentration would allow a more even distribution of mPEG within the nose, while remaining viscous enough to stay within the nasal cavity for the 3-5 minutes required for cell modification (135).

Importantly, our finding that RSV infection can be inhibited by mPEG-modification of the host cells provides another example of a virus that can be inhibited by this strategy. This further strengthens the use of mPEG modification of the nasal epithelium as a broad-spectrum anti-viral prophylactic, and future studies should evaluate this strategy *in vivo*.

6.2.3 Summary of Discussion on mPEG-Modified Host Cells

In summary, we found that mPEG-modification of the host cells prevented RSV infection. This finding confirms our hypothesis and is consistent with previous studies on mPEG-modification of host cells. We found that grafting of long polymers particularly at high grafting concentrations resulted in a dramatic decrease in infection and provided near complete protection against RSV. However, grafting of short polymers provided virtually no protection. We postulate that these clear differences in efficacy with different polymer lengths relate to

properties of the RSV receptor, specifically, its size, chemical composition, and surrounding microenvironment. Furthermore, given that long chain polymers prevent RSV infection over a broad range of virus doses, and in both polarized and non-polarized cells, our findings indicate that mPEG-modification of the host cells will most likely be effective *in vivo*. In combination with previous studies showing that host cell modification prevents against infection by other respiratory viruses, our findings strongly indicate that mPEG-modification of the nasal epithelium will be an effective broad-spectrum anti-viral prophylactic therapy.

6.3 mPEG-MODIFICATION OF RSV OR ITS HOST CELL: "TARGET SIZE DICTATES POLYMER SIZE"

Our findings support both of our hypotheses that mPEG-modification of RSV and mPEG-modification the host cells would prevent infection. Importantly, we found distinct differences in the efficacy of both strategies with different polymer lengths. For mPEG-modification of the virus, we found that grafting of short (2 kDa) chain polymers provided enhanced protection relative to longer (20 kDa) polymers. In contrast, for mPEG-modification of the host cells, grafting of long chain (20 kDa) polymers protected host cells from RSV, while short (2 kDa) polymers did not prevent infection. Thus, given the difference in size between RSV and HeLa cells (150-300 nm and 11-24 μm in diameter, respectively) (4, 193), our findings indicate that the length of polymer required to protect the surface of a particle should be tailored to the size of the particle. This is a novel finding and may lead to enhanced efficacy of mPEG modification strategies with other pathogens (*i.e.* bacteria) or with cells and cell fragments (*i.e.* red blood cells and platelets).

Comparing the two PEGylation strategies, at equivalent polymer lengths mPEG-modification of the virus results in a greater reduction in infection than mPEG-modification of

the host cells. For example, with an MOI of 0.5 and grafting concentration of 7.5 mM, at 72 hours post-infection 20 kDa mPEG modification of RSV decreases infection by 88.6%, while modification of the host cell decreases infection by 45%. This may be the result of mPEG being able to form a more dense layer on the smaller virus surface relative to the host cell surface. Alternatively, this may be the result of differences in the ability of mPEG-modified RSV to more greatly inhibit interactions with the host cell.

As previously mentioned, HS is a prominent feature on the host cell surface extending 40-160 nm (82), and mPEG-modification of the virus will both physically and electrostatically inhibit RSV from binding to HS. In contrast, on the host cell surface, HS is not readily modified and will likely extend beyond the 50 nm, 20 kDa mPEG zone of exclusion. Therefore, on mPEG-modified cells, RSV will likely be able to bind and interact with HS. Subsequently, HS may facilitate RSV in finding an unmodified receptor, and hence, may result in a slightly lower efficacy with mPEG-modification of the cells compared to mPEG-modification of the virus.

6.4 CONCLUSIONS AND OVERALL SIGNIFICANCE OF FINDINGS

In conclusion, both mPEG-modification of RSV and the host cell are highly effective strategies for preventing RSV infection. The overall significance of our findings is that to provide optimal protection, polymer length should be tailored to the size of particle being modified. Therefore, when modifying large particles such as cells or tissue, large polymers should be used. In contrast, when modifying small particles such as viruses, bacteria, or platelets, short polymers will provide enhanced protection.

In the context of enveloped viruses, viruses such as pandemic influenza A and SARS-coronavirus are re-emerging or emerging and present a serious risk to human health (194-196). Our results indicate that mPEG-modification may also be effective against these viruses. For example, influenza A has hemagglutinin spikes on its surface that are analogous to the F protein

spikes on RSV (194, 195). Therefore, mPEG-modification of influenza A may similarly block infection and provide another vaccine strategy against influenza. Alternatively, given the threat of theses viruses and the time required to produce a vaccine, modification of the upper respiratory tract may also be protective against influenza and may provide an effective prophylactic in the event of a pandemic outbreak.

Given that both PEGylation strategies show promise as effective anti-RSV prophylactics and may be effective against other enveloped viruses, future *in vivo* studies should evaluate mPEG-modification as a vaccine strategy and as a nasal spray. Importantly, the results of these studies may lead to the development of novel cost-effective prophylactics, capable of diminishing the disease burden of RSV and other respiratory viruses.

REFERENCES

1. Blount RE, Jr., Morris JA, Savage RE. Recovery of cytopathogenic agent from chimpanzees with coryza. Proc Soc Exp Biol Med. 1956;92:544-549.
2. Chanock R, Roizman B, Myers R. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). I. Isolation, properties and characterization. Am J Hyg. 1957;66:281-290.
3. Chanock RM, Kim HW, Vargosko AJ et al. Respiratory syncytial virus. I. Virus recovery and other observations during 1960 outbreak of bronchiolitis, pneumonia, and minor respiratory diseases in children. JAMA. 1961;176:647-653.
4. Collins PL, Crowe JE, Jr. Respiratory Syncytial Virus and Metapneumovirus. In: Knipe DM, Griffin DE, Lamb RA, Straus SE, Howley PM, Martin MA, Roizman B, editors. Fields Virology. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 1601-1646.
5. Henderson FW, Collier AM, Clyde WAJ, Denny FW. Respiratory-syncytial-virus infections, reinfections and immunity. A prospective, longitudinal study in young children. N Engl J Med. 1979;300:530-534.
6. Hall CB, Walsh EE, Long CE, Schnabel KC. Immunity to and frequency of reinfection with respiratory syncytial virus. J Infect Dis. 1991;163:693-698.
7. Hall CB, Long CE, Schnabel KC. Respiratory syncytial virus infections in previously healthy working adults. Clin Infect Dis. 2001;33:792-796.
8. Glezen WP, Taber LH, Frank AL, Kasel JA. Risk of primary infection and reinfection with respiratory syncytial virus. Am J Dis Child. 1986;140:543-546.
9. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. N Engl J Med. 2005;352:1749-1759.

10. Falsey AR, Walsh EE. Respiratory syncytial virus infection in elderly adults. Drugs Aging. 2005;22:577-587.

11. Ghosh S, Champlin RE, Englund J et al. Respiratory syncytial virus upper respiratory tract illnesses in adult blood and marrow transplant recipients: combination therapy with aerosolized ribavirin and intravenous immunoglobulin. Bone Marrow Transplant. 2000;25:751-755.

12. Englund JA, Sullivan CJ, Jordan MC, Dehner LP, Vercellotti GM, Balfour HH, Jr. Respiratory syncytial virus infection in immunocompromised adults. Ann Intern Med. 1988;109:203-208.

13. Englund JA, Anderson LJ, Rhame FS. Nosocomial transmission of respiratory syncytial virus in immunocompromised adults. J Clin Microbiol. 1991;29:115-119.

14. Englund JA, Piedra PA, Whimbey E. Prevention and treatment of respiratory syncytial virus and parainfluenza viruses in immunocompromised patients. Am J Med. 1997;102:61-70; discussion 75-6.

15. Whimbey E, Couch RB, Englund JA et al. Respiratory syncytial virus pneumonia in hospitalized adult patients with leukemia. Clin Infect Dis. 1995;21:376-379.

16. McColl MD, Corser RB, Bremner J, Chopra R. Respiratory syncytial virus infection in adult BMT recipients: effective therapy with short duration nebulised ribavirin. Bone Marrow Transplant. 1998;21:423-425.

17. Wendt CH. Community respiratory viruses: organ transplant recipients. Am J Med. 1997;102:31-6; discussion 42-3.

18. McCarthy AJ, Kingman HM, Kelly C et al. The outcome of 26 patients with respiratory syncytial virus infection following allogeneic stem cell transplantation. Bone Marrow Transplant. 1999;24:1315-1322.

19. Flynn JD, Akers WS, Jones M et al. Treatment of respiratory syncytial virus pneumonia in a lung transplant recipient: case report and review of the literature. Pharmacotherapy. 2004;24:932-938.

20. Hall CB. Respiratory syncytial virus and parainfluenza virus. N Engl J Med. 2001;344:1917-1928.

21. Goodman D. Bronchiolitis. In: Behrman RE, Kliegman, R.M., Jenson, H.B., editor. Nelson Textbook of Pediatrics, 17th Edition. Philadelphia, PA: W.B. Saunders Company; 2004. p. 1415.

22. Domachowske JB, Rosenberg HF. Respiratory syncytial virus infection: immune response, immunopathogenesis, and treatment. Clin Microbiol Rev. 1999;12:298-309.

23. Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ. Bronchiolitis-associated hospitalizations among US children, 1980-1996. JAMA. 1999;282:1440-1446.

24. Paramore LC, Ciuryla V, Ciesla G, Liu L. Economic impact of respiratory syncytial virus-related illness in the US: an analysis of national databases. Pharmacoeconomics. 2004;22:275-284.

25. Leader S, Kohlhase K. Recent trends in severe respiratory syncytial virus (RSV) among US infants, 1997 to 2000. J Pediatr. 2003;143:S127-32.

26. Handforth J, Friedland JS, Sharland M. Basic epidemiology and immunopathology of RSV in children. Paediatr Respir Rev. 2000;1:210-214.

27. Boyce TG, Mellen BG, Mitchel EF, Jr., Wright PF, Griffin MR. Rates of hospitalization for respiratory syncytial virus infection among children in medicaid. J Pediatr. 2000;137:865-870.

28. Welliver RC. Review of epidemiology and clinical risk factors for severe respiratory syncytial virus (RSV) infection. J Pediatr. 2003;143:S112-7.

29. Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. Am J Respir Crit Care Med. 2000;161:1501-1507.

30. Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B, Bjorksten B. Asthma and immunoglobulin E antibodies after respiratory syncytial virus bronchiolitis: a prospective cohort study with matched controls. Pediatrics. 1995;95:500-505.

31. Sigurs N, Gustafsson PM, Bjarnason R et al. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. Am J Respir Crit Care Med. 2005;171:137-141.

32. Hashem M, Hall CB. Respiratory syncytial virus in healthy adults: the cost of a cold. J Clin Virol. 2003;27:14-21.

33. Ghosh S, Champlin RE, Ueno NT et al. Respiratory syncytial virus infections in autologous blood and marrow transplant recipients with breast cancer: combination therapy with aerosolized ribavirin and parenteral immunoglobulins. Bone Marrow Transplant. 2001;28:271-275.

34. King JCJ. Community respiratory viruses in individuals with human immunodeficiency virus infection. Am J Med. 1997;102:19-24; discussion 25-6.

35. Hanson C, Shearer WT. Bronchiolitis. In: McMillan J. A. DCD, Feigin R.D., and Warshaw J.B., editor. Oski's Pediatrics: Principles and Practice 3rd Edition. Philadelphia: Lippincott Williams & Wilkins; 1999.

36. Welliver RC. Respiratory syncytial virus infection: therapy and prevention. Paediatr Respir Rev. 2004;5 Suppl A:S127-33.

37. Black CP. Systematic review of the biology and medical management of respiratory syncytial virus infection. Respir Care. 2003;48:209-31; discussion 231-3.

38. Langley JM, Smith MB, LeBlanc JC, Joudrey H, Ojah CR, Pianosi P. Racemic epinephrine compared to salbutamol in hospitalized young children with bronchiolitis; a randomized controlled clinical trial [ISRCTN46561076]. BMC Pediatr. 2005;5:7.

39. Wainwright C, Altamirano L, Cheney M et al. A multicenter, randomized, double-blind, controlled trial of nebulized epinephrine in infants with acute bronchiolitis. N Engl J Med. 2003;349:27-35.

40. Richter H, Seddon P. Early nebulized budesonide in the treatment of bronchiolitis and the prevention of postbronchiolitic wheezing. J Pediatr. 1998;132:849-853.

41. Roosevelt G, Sheehan K, Grupp-Phelan J, Tanz RR, Listernick R. Dexamethasone in bronchiolitis: a randomised controlled trial. Lancet. 1996;348:292-295.

42. Bulow SM, Nir M, Levin E et al. Prednisolone treatment of respiratory syncytial virus infection: a randomized controlled trial of 147 infants. Pediatrics. 1999;104:e77.

43. Chidgey SM, Broadley KJ. Respiratory syncytial virus infections: characteristics and treatment. J Pharm Pharmacol. 2005;57:1371-1381.

44. Collins CL, Pollard AJ. Respiratory syncytial virus infections in children and adults. J Infect. 2002;45:10-17.

45. Reassessment of the indications for ribavirin therapy in respiratory syncytial virus infections. American Academy of Pediatrics Committee on Infectious Diseases. Pediatrics. 1996;97:137-140.

46. Kim HW, Canchola JG, Brandt CD et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol. 1969;89:422-434.

47. Dudas RA, Karron RA. Respiratory syncytial virus vaccines. Clin Microbiol Rev. 1998;11:430-439.

48. McKay E, Higgins P, Tyrrell D, Pringle C. Immunogenicity and pathogenicity of temperature-sensitive modified respiratory syncytial virus in adult volunteers. J Med Virol. 1988;25:411-421.

49. Pringle CR, Filipiuk AH, Robinson BS, Watt PJ, Higgins P, Tyrrell DA. Immunogenicity and pathogenicity of a triple temperature-sensitive modified respiratory syncytial virus in adult volunteers. Vaccine. 1993;11:473-478.

50. Falsey AR, Walsh EE. Safety and immunogenicity of a respiratory syncytial virus subunit vaccine (PFP-2) in the institutionalized elderly. Vaccine. 1997;15:1130-1132.

51. Falsey AR, Walsh EE. Safety and immunogenicity of a respiratory syncytial virus subunit vaccine (PFP-2) in ambulatory adults over age 60. Vaccine. 1996;14:1214-1218.

52. Munoz FM, Piedra PA, Glezen WP. Safety and immunogenicity of respiratory syncytial virus purified fusion protein-2 vaccine in pregnant women. Vaccine. 2003;21:3465-3467.

53. Groothuis JR, King SJ, Hogerman DA, Paradiso PR, Simoes EA. Safety and immunogenicity of a purified F protein respiratory syncytial virus (PFP-2) vaccine in seropositive children with bronchopulmonary dysplasia. J Infect Dis. 1998;177:467-469.

54. Piedra PA, Cron SG, Jewell A et al. Immunogenicity of a new purified fusion protein vaccine to respiratory syncytial virus: a multi-center trial in children with cystic fibrosis. Vaccine. 2003;21:2448-2460.

55. Meyer G, Deplanche M, Schelcher F. Human and bovine respiratory syncytial virus vaccine research and development. Comp Immunol Microbiol Infect Dis. 2008;31:191-225.

56. Reduction of respiratory syncytial virus hospitalization among premature infants and infants with bronchopulmonary dysplasia using respiratory syncytial virus immune globulin prophylaxis. The PREVENT Study Group. Pediatrics. 1997;99:93-99.

57. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. The IMpact-RSV Study Group. Pediatrics. 1998;102:531-537.

58. Meissner HC, Long SS. Revised indications for the use of palivizumab and respiratory syncytial virus immune globulin intravenous for the prevention of respiratory syncytial virus infections. Pediatrics. 2003;112:1447-1452.

59. Meissner HC, Long SS. Revised indications for the use of palivizumab and respiratory syncytial virus immune globulin intravenous for the prevention of respiratory syncytial virus infections. Pediatrics. 2003;112:1447-1452.

60. Smith S. Availability of Synagis(Palivizumab). Canadian Blood Services Customer Letter #2007-33. [Online]. 2007 [cited 2008 Dec 22]; Available from: URL: http://www.bloodservices.ca.

61. Musher DM. How contagious are common respiratory tract infections? N Engl J Med. 2003;348:1256-1266.

62. Chavez-Bueno S, Mejias A, Welliver RC. Respiratory syncytial virus bronchiolitis : current and future strategies for treatment and prophylaxis. Treat Respir Med. 2006;5:483-494.

63. American Tissue Culture Collection Technical Services. Animal Virology Frequently Asked Questions. [Online]. 2009 [cited 2009 Feb 12]; Available from URL: http://www.atcc.org.

64. Kravetz HM, Knight V, Chanock RM et al. Respiratory syncytial virus. III. Production of illness and clinical observations in adult volunteers. JAMA. 1961;176:657-663.

65. Devincenzo JP. Natural infection of infants with respiratory syncytial virus subgroups A and B: a study of frequency, disease severity, and viral load. Pediatr Res. 2004;56:914-917.

66. Techaarpornkul S, Barretto N, Peeples ME. Functional analysis of recombinant respiratory syncytial virus deletion mutants lacking the small hydrophobic and/or attachment glycoprotein gene. J Virol. 2001;75:6825-6834.

67. Jin H, Zhou H, Cheng X, Tang R, Munoz M, Nguyen N. Recombinant respiratory syncytial viruses with deletions in the NS1, NS2, SH, and M2-2 genes are attenuated in vitro and in vivo. Virology. 2000;273:210-218.

68. Whitehead SS, Bukreyev A, Teng MN et al. Recombinant respiratory syncytial virus bearing a deletion of either the NS2 or SH gene is attenuated in chimpanzees. J Virol. 1999;73:3438-3442.

69. Teng MN, Whitehead SS, Bermingham A et al. Recombinant respiratory syncytial virus that does not express the NS1 or M2-2 protein is highly attenuated and immunogenic in chimpanzees. J Virol. 2000;74:9317-9321.

70. Spann KM, Tran KC, Chi B, Rabin RL, Collins PL. Suppression of the induction of alpha, beta, and lambda interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected]. J Virol. 2004;78:4363-4369.

71. Spann KM, Tran KC, Collins PL. Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. J Virol. 2005;79:5353-5362.

72. Brown G, Rixon HW, Sugrue RJ. Respiratory syncytial virus assembly occurs in GM1-rich regions of the host-cell membrane and alters the cellular distribution of tyrosine phosphorylated caveolin-1. J Gen Virol. 2002;83:1841-1850.

73. Brown G, Aitken J, Rixon HW, Sugrue RJ. Caveolin-1 is incorporated into mature respiratory syncytial virus particles during virus assembly on the surface of virus-infected cells. J Gen Virol. 2002;83:611-621.

74. Brown G, Jeffree CE, McDonald T, Rixon HW, Aitken JD, Sugrue RJ. Analysis of the interaction between respiratory syncytial virus and lipid-rafts in Hep2 cells during infection. Virology. 2004;327:175-185.

75. Harris J, Werling D. Binding and entry of respiratory syncytial virus into host cells and initiation of the innate immune response. Cell Microbiol. 2003;5:671-680.

76. Zhang L, Peeples ME, Boucher RC, Collins PL, Pickles RJ. Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. J Virol. 2002;76:5654-5666.

77. Roberts SR, Compans RW, Wertz GW. Respiratory syncytial virus matures at the apical surfaces of polarized epithelial cells. J Virol. 1995;69:2667-2673.

78. Wagner EK, Hewlett MJ. Replication strategies of RNA viruses requiring RNA-directed mRNA transcription as the first step in viral gene expression. Basic Virology. Maden, MA: Blackwell Publishing; 2004. p. 257-285.

79. Levine S, Hamilton R. Kinetics of the respiratory syncytial virus growth cycle in HeLa cells. Arch Gesamte Virusforsch. 1969;28:122-132.

80. Tristram DA, Hicks WJ, Hard R. Respiratory syncytial virus and human bronchial epithelium. Arch Otolaryngol Head Neck Surg. 1998;124:777-783.

81. Hallak LK, Spillmann D, Collins PL, Peeples ME. Glycosaminoglycan sulfation requirements for respiratory syncytial virus infection. J Virol. 2000;74:10508-10513.

82. Rabenstein DL. Heparin and heparan sulfate: structure and function. Nat Prod Rep. 2002;19:312-331.

83. Feldman SA, Hendry RM, Beeler JA. Identification of a linear heparin binding domain for human respiratory syncytial virus attachment glycoprotein G. J Virol. 1999;73:6610-6617.

84. Crim RL, Audet SA, Feldman SA, Mostowski HS, Beeler JA. Identification of linear heparin-binding peptides derived from human respiratory syncytial virus fusion glycoprotein that inhibit infectivity. J Virol. 2007;81:261-271.

85. Harcourt J, Alvarez R, Jones LP, Henderson C, Anderson LJ, Tripp RA. Respiratory syncytial virus G protein and G protein CX3C motif adversely affect CX3CR1+ T cell responses. J Immunol. 2006;176:1600-1608.

86. Kurt-Jones EA, Popova L, Kwinn L et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol. 2000;1:398-401.

87. Pastey MK, Crowe JEJ, Graham BS. RhoA interacts with the fusion glycoprotein of respiratory syncytial virus and facilitates virus-induced syncytium formation. J Virol. 1999;73:7262-7270.

88. Malhotra R, Ward M, Bright H et al. Isolation and characterisation of potential respiratory syncytial virus receptor(s) on epithelial cells. Microbes Infect. 2003;5:123-133.

89. Bachi T. Direct observation of the budding and fusion of an enveloped virus by video microscopy of viable cells. J Cell Biol. 1988;107:1689-1695.

90. Monick MM, Yarovinsky TO, Powers LS et al. Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. J Biol Chem. 2003;278:53035-53044.

91. Arnold R, Humbert B, Werchau H, Gallati H, Konig W. Interleukin-8, interleukin-6, and soluble tumour necrosis factor receptor type I release from a human pulmonary epithelial cell line (A549) exposed to respiratory syncytial virus. Immunology. 1994;82:126-133.

92. Bitko V, Velazquez A, Yang L, Yang YC, Barik S. Transcriptional induction of multiple cytokines by human respiratory syncytial virus requires activation of NF-kappa B and is inhibited by sodium salicylate and aspirin. Virology. 1997;232:369-378.

93. Olszewska-Pazdrak B, Casola A, Saito T et al. Cell-specific expression of RANTES, MCP-1, and MIP-1alpha by lower airway epithelial cells and eosinophils infected with respiratory syncytial virus. J Virol. 1998;72:4756-4764.

94. Openshaw PJ, Tregoning JS. Immune responses and disease enhancement during respiratory syncytial virus infection. Clin Microbiol Rev. 2005;18:541-555.

95. Glass WG, Rosenberg HF, Murphy PM. Chemokine regulation of inflammation during acute viral infection. Curr Opin Allergy Clin Immunol. 2003;3:467-473.

96. Van Damme J, Proost P, Lenaerts JP, Opdenakker G. Structural and functional identification of two human, tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonging to the chemokine family. J Exp Med. 1992;176:59-65.

97. Lee SC, Brummet ME, Shahabuddin S et al. Cutaneous injection of human subjects with macrophage inflammatory protein-1 alpha induces significant recruitment of neutrophils and monocytes. J Immunol. 2000;164:3392-3401.

98. Jamaluddin M, Wang S, Garofalo RP et al. IFN-beta mediates coordinate expression of antigen-processing genes in RSV-infected pulmonary epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2001;280:L248-57.

99. van Drunen Littel-van den Hurk S, Mapletoft JW, Arsic N, Kovacs-Nolan J. Immunopathology of RSV infection: prospects for developing vaccines without this complication. Rev Med Virol. 2007;17:5-34.

100. Alam R, Stafford S, Forsythe P et al. RANTES is a chemotactic and activating factor for human eosinophils. J Immunol. 1993;150:3442-3448.

101. Guerrero-Plata A, Casola A, Suarez G et al. Differential response of dendritic cells to human metapneumovirus and respiratory syncytial virus. Am J Respir Cell Mol Biol. 2006;34:320-329.

102. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol. 1986;136:2348-2357.

103. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today. 1996;17:138-146.

104. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol. 1989;7:145-173.

105. Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD, Allen JE. Helminth parasites--masters of regulation. Immunol Rev. 2004;201:89-116.

106. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 Family Cytokines and the Expanding Diversity of Effector T Cell Lineages. Annu Rev Immunol. 2007

107. Aberle JH, Aberle SW, Dworzak MN et al. Reduced interferon-gamma expression in peripheral blood mononuclear cells of infants with severe respiratory syncytial virus disease. Am J Respir Crit Care Med. 1999;160:1263-1268.

108. Legg JP, Hussain IR, Warner JA, Johnston SL, Warner JO. Type 1 and type 2 cytokine imbalance in acute respiratory syncytial virus bronchiolitis. Am J Respir Crit Care Med. 2003;168:633-639.

109. Abuchowski A, McCoy JR, Palczuk NC, van Es T, Davis FF. Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. J Biol Chem. 1977;252:3582-3586.

110. Abuchowski A, van Es T, Palczuk NC, Davis FF. Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. J Biol Chem. 1977;252:3578-3581.

111. Scott MD, Murad KL, Koumpouras F, Talbot M, Eaton JW. Chemical camouflage of antigenic determinants: stealth erythrocytes. Proc Natl Acad Sci U S A. 1997;94:7566-7571.

112. Chen AM, Scott MD. Current and future applications of immunological attenuation via pegylation of cells and tissue. BioDrugs. 2001;15:833-847.

113. Scott MD, Murad KL. Cellular camouflage: fooling the immune system with polymers. Curr Pharm Des. 1998;4:423-438.

114. Chen AM, Scott MD. Immunocamouflage: prevention of transfusion-induced graft-versus-host disease via polymer grafting of donor cells. J Biomed Mater Res A. 2003;67:626-636.

115. O'Riordan CR, Lachapelle A, Delgado C et al. PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. Hum Gene Ther. 1999;10:1349-1358.

116. McCoy LL, Scott MD. Broad-Spectrum Antiviral Prophylaxis: Inhibition of Viral Infection by Polymer Grafting with Methoxypoly(ethylene glycol). In: PF T, editor. Antiviral Drug Discovery for Emerging Diseases and Bioterrorism Threats. John Wiley & Sons, Inc.; 2005. p. 379-395.

117. Roberts MJ, Bentley MD, Harris JM. Chemistry for peptide and protein PEGylation. Adv Drug Deliv Rev. 2002;54:459-476.

118. Veronese FM. Peptide and protein PEGylation: a review of problems and solutions. Biomaterials. 2001;22:405-417.

119. Laysan Bio Incoporated Products Page: mPEG-SVA_final-review. [Online]. 2009 [cited 2009 Feb 23]; Available from: URL: http://laysanbio.com.

120. Jackson CJ, Charlton JL, Kuzminski K, Lang GM, Sehon AH. Synthesis, isolation, and characterization of conjugates of ovalbumin with monomethoxypolyethylene glycol using cyanuric chloride as the coupling agent. Anal Biochem. 1987;165:114-127.

121. Shearwater Polymers Inc. Functionalized biocompatible polymers for research and pharmaceuticals: Polyethylene glycol and derivatives. Huntsville, AL: Shearwater Polymers Inc; 2001. p 1-50.

122. Bailon P, Palleroni A, Schaffer CA et al. Rational design of a potent, long-lasting form of interferon: a 40 kDa branched polyethylene glycol-conjugated interferon alpha-2a for the treatment of hepatitis C. Bioconjug Chem. 2001;12:195-202.

123. Monkarsh SP, Ma Y, Aglione A et al. Positional isomers of monopegylated interferon alpha-2a: isolation, characterization, and biological activity. Anal Biochem. 1997;247:434-440.

124. Lee H, Park TG. Preparation and characterization of mono-PEGylated epidermal growth factor: evaluation of in vitro biologic activity. Pharm Res. 2002;19:845-851.

125. Lehninger AL, Nelson DL, Cox MM. Lehninger principles of biochemistry. Freeman; 2004

126. Scott MD, Chen AM. Beyond the red cell: pegylation of other blood cells and tissues. Transfus Clin Biol. 2004;11:40-46.

127. Antonsen KP. Water Structure of PEG Solutions by Differential Scanning Calorimetry Measurements. In: Harris MJ, editor. Poly(ethylene glycol) Chemistry. New York, NY: Plenum Press; 1992. p. 15-28.

128. de Gennes PG. Conformations of Polymers Attached to an Interface. Macromolecules. 1980;13:1069-1075.

129. Allen C, Dos Santos N, Gallagher R et al. Controlling the physical behavior and biological performance of liposome formulations through use of surface grafted poly(ethylene glycol). Biosci Rep. 2002;22:225-250.

130. Bradley AJ, Test ST, Murad KL, Mitsuyoshi J, Scott MD. Interactions of IgM ABO antibodies and complement with methoxy-PEG-modified human RBCs. Transfusion. 2001;41:1225-1233.

131. Carignano MA, Szleifer II. Prevention of protein adsorption by flexible and rigid chain molecules. Colloids Surf B Biointerfaces. 2000;18:169-182.

132. Satulovsky J, Carignano MA, Szleifer I. Kinetic and thermodynamic control of protein adsorption. Proc Natl Acad Sci U S A. 2000;97:9037-9041.

133. Kenworthy AK, Hristova K, Needham D, McIntosh TJ. Range and magnitude of the steric pressure between bilayers containing phospholipids with covalently attached poly(ethylene glycol). Biophys J. 1995;68:1921-1936.

134. Chen AM, Scott MD. Comparative analysis of polymer and linker chemistries on the efficacy of immunocamouflage of murine leukocytes. Artif Cells Blood Substit Immobil Biotechnol. 2006;34:305-322.

135. McCoy LL. Prevention of Viral Infection via Modification of Virus or Cells with Methoxypoly(ethylene glycol). [dissertation]. Vancouver, BC, Canada: University of British Columbia; 2005.

136. Delgado C, Francis GE, Fisher D. The uses and properties of PEG-linked proteins. Crit Rev Ther Drug Carrier Syst. 1992;9:249-304.

137. Yamaoka T, Tabata Y, Ikada Y. Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration to mice. J Pharm Sci. 1994;83:601-606.

138. Working PK, Newman MS, Johnson J, Cornacoff JB. Safety of Poly(ethylene glycol) and Poly(ethylene glycol) Derivatives. In: Harris MJ, Zalipsky S, editors. Poly(ethylene glycol) Chemistry and Biological Applications. Washington, DC: American Chemical Society; 1997.

139. Richter AW, Akerblom E. Antibodies against polyethylene glycol produced in animals by immunization with monomethoxy polyethylene glycol modified proteins. Int Arch Allergy Appl Immunol. 1983;70:124-131.

140. Richter AW, Akerblom E. Polyethylene glycol reactive antibodies in man: titer distribution in allergic patients treated with monomethoxy polyethylene glycol modified allergens or placebo, and in healthy blood donors. Int Arch Allergy Appl Immunol. 1984;74:36-39.

141. Armstrong JK, Hempel G, Koling S et al. Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. Cancer. 2007;110:103-111.

142. Ganson NJ, Kelly SJ, Scarlett E, Sundy JS, Hershfield MS. Control of hyperuricemia in subjects with refractory gout, and induction of antibody against poly(ethylene glycol) (PEG), in a phase I trial of subcutaneous PEGylated urate oxidase. Arthritis Res Ther. 2006;8:R12.

143. Croyle MA, Chirmule N, Zhang Y, Wilson JM. "Stealth" adenoviruses blunt cell-mediated and humoral immune responses against the virus and allow for significant gene expression upon readministration in the lung. J Virol. 2001;75:4792-4801.

144. Kreppel F, Kochanek S. Modification of adenovirus gene transfer vectors with synthetic polymers: a scientific review and technical guide. Mol Ther. 2008;16:16-29.

145. Murad KL, Mahany KL, Brugnara C, Kuypers FA, Eaton JW, Scott MD. Structural and functional consequences of antigenic modulation of red blood cells with methoxypoly(ethylene glycol). Blood. 1999;93:2121-2127.

146. Hershfield MS. Biochemistry and Immunology of Poly(ethylene glycol)-Modified Adenosine Deaminase (PEG-ADA). In: Harris MJ, Zalipsky S, editors. Poly(ethylene glycol) Chemistry and Biological Applications. Washington, DC: American Chemical Society; 1997. p. 145-154.

147. Veronese FM, Schiavon O, Pasut G et al. PEG-doxorubicin conjugates: influence of polymer structure on drug release, in vitro cytotoxicity, biodistribution, and antitumor activity. Bioconjug Chem. 2005;16:775-784.

148. Kim TH, Lee H, Park TG. Pegylated recombinant human epidermal growth factor (rhEGF) for sustained release from biodegradable PLGA microspheres. Biomaterials. 2002;23:2311-2317.

149. Rostin J, Smeds AL, Akerblom E. B-Domain deleted recombinant coagulation factor VIII modified with monomethoxy polyethylene glycol. Bioconjug Chem. 2000;11:387-396.

150. Park YK, Abuchowski A, Davis S, Davis F. Pharmacology of Escherichia coli-L-asparaginase polyethylene glycol adduct. Anticancer Res. 1981;1:373-376.

151. Kimura M, Matsumura Y, Miyauchi Y, Maeda H. A new tactic for the treatment of jaundice: an injectable polymer-conjugated Bilirubin oxidase. Proc Soc Exp Biol Med. 1988;188:364-369.

152. Goodson RJ, Katre NV. Site-directed pegylation of recombinant interleukin-2 at its glycosylation site. Biotechnology (N Y). 1990;8:343-346.

153. Jackson RM, Veal CF, Beckman JS, Brannen AL. Polyethylene glycol-conjugated superoxide dismutase in unilateral lung injury due to re-expansion (re-oxygenation). Am J Med Sci. 1990;300:22-28.

154. Kita Y, Rohde MF, Arakawa T, Fagin KD, Fish EN, Banerjee K. Characterization of a polyethylene glycol conjugate of recombinant human interferon-gamma. Drug Des Deliv. 1990;6:157-167.

155. Nakagomi K, Ajisaka K. Chemical modification of alpha-thrombin and in vitro characterization of its anticoagulant activity. Biochem Int. 1990;22:75-84.

156. Avramis VI, Sencer S, Periclou AP et al. A randomized comparison of native Escherichia coli asparaginase and polyethylene glycol conjugated asparaginase for treatment of children with newly diagnosed standard-risk acute lymphoblastic leukemia: a Children's Cancer Group study. Blood. 2002;99:1986-1994.

157. Soares AL, Guimaraes GM, Polakiewicz B, de Moraes Pitombo RN, Abrahao-Neto J. Effects of polyethylene glycol attachment on physicochemical and biological stability of E. coli L-asparaginase. Int J Pharm. 2002;237:163-170.

158. Hoffman RM. PEG-methioninase. Adv Exp Med Biol. 2003;519:69-79.

159. Pasut G, Canal F, Dalla Via L, Arpicco S, Veronese FM, Schiavon O. Antitumoral activity of PEG-gemcitabine prodrugs targeted by folic acid. J Control Release. 2008;127:239-248.

160. McClean PL, Irwin N, Hunter K, Gault VA, Flatt PR. (Pro(3))GIP[mPEG]: novel, long-acting, mPEGylated antagonist of gastric inhibitory polypeptide for obesity-diabetes (diabesity) therapy. Br J Pharmacol. 2008;155:690-701.

161. Bradley AJ, Scott MD. Separation and purification of methoxypoly(ethylene glycol) grafted red blood cells via two-phase partitioning. J Chromatogr B Analyt Technol Biomed Life Sci. 2004;807:163-168.

162. Bradley AJ, Scott MD. Immune complex binding by immunocamouflaged [poly(ethylene glycol)-grafted] erythrocytes. Am J Hematol. 2007;82:970-975.

163. Bradley AJ, Murad KL, Regan KL, Scott MD. Biophysical consequences of linker chemistry and polymer size on stealth erythrocytes: size does matter. Biochim Biophys Acta. 2002;1561:147-158.

164. Murad KL, Gosselin EJ, Eaton JW, Scott MD. Stealth cells: prevention of major histocompatibility complex class II-mediated T-cell activation by cell surface modification. Blood. 1999;94:2135-2141.

165. Lee DY, Yang K, Lee S et al. Optimization of monomethoxy-polyethylene glycol grafting on the pancreatic islet capsules. J Biomed Mater Res. 2002;62:372-377.

166. Lee DY, Nam JH, Byun Y. Effect of polyethylene glycol grafted onto islet capsules on prevention of splenocyte and cytokine attacks. J Biomater Sci Polym Ed. 2004;15:753-766.

167. Jang JY, Lee DY, Park SJ, Byun Y. Immune reactions of lymphocytes and macrophages against PEG-grafted pancreatic islets. Biomaterials. 2004;25:3663-3669.

168. Croyle MA, Yu QC, Wilson JM. Development of a rapid method for the PEGylation of adenoviruses with enhanced transduction and improved stability under harsh storage conditions. Hum Gene Ther. 2000;11:1713-1722.

169. Mizouni SK, Lehman JM, Cohen B, Scott MD. Viral modification with methoxypoly(ethylene glycol): Implications for gene therapy and viral inactivation. Blood. 1998;92:4627.

170. Mizouni SK, Bradley AJ, Scott MD. Use of a two-phase partitioning system to purify an immunologically attenuated viral vector. Blood. 1999;94:5081 (415b).

171. Mizouni S, Scott MD. Viral modification with methoxypoly(ethylene glycol):implication for viral inactivation and gene therapy? [dissertation]. Albany: 2000.

172. Fields BN, Knipe DM, Howley PM. Fields' virology. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2007:2 v.

173. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. Structure of the human class I histocompatibility antigen, HLA-A2. Nature. 1987;329:506-512.

174. He Y, Chipman PR, Howitt J et al. Interaction of coxsackievirus B3 with the full length coxsackievirus-adenovirus receptor. Nat Struct Biol. 2001;8:874-878.

175. Hussain MM, Tranum-Jensen J, Noren O, Sjostrom H, Christiansen K. Reconstitution of purified amphiphilic pig intestinal microvillus aminopeptidase. Mode of membrane insertion and morphology. Biochem J. 1981;199:179-186.

176. Mi LZ, Grey MJ, Nishida N, Walz T, Lu C, Springer TA. Functional and structural stability of the epidermal growth factor receptor in detergent micelles and phospholipid nanodiscs. Biochemistry. 2008;47:10314-10323.

177. Jun CD, Carman CV, Redick SD, Shimaoka M, Erickson HP, Springer TA. Ultrastructure and function of dimeric, soluble intercellular adhesion molecule-1 (ICAM-1). J Biol Chem. 2001;276:29019-29027.

178. Scott MD. Enhancement of oligomeric viral immunogenicity. Albany Medical College (Albany, NY). 2003;United States:1-17.

179. Scott MD. Covalent attachment of polymer to cell to prevent virus bonding to receptor. Albany Medical College (Albany, NY). 2004;United States:1-26.

180. Hosoya M, Balzarini J, Shigeta S, De Clercq E. Differential inhibitory effects of sulfated polysaccharides and polymers on the replication of various myxoviruses and retroviruses, depending on the composition of the target amino acid sequences of the viral envelope glycoproteins. Antimicrob Agents Chemother. 1991;35:2515-2520.

181. Bolt G, Pedersen LO, Birkeslund HH. Cleavage of the respiratory syncytial virus fusion protein is required for its surface expression: role of furin. Virus Res. 2000;68:25-33.

182. Russell CJ, Jardetzky TS, Lamb RA. Membrane fusion machines of paramyxoviruses: capture of intermediates of fusion. EMBO J. 2001;20:4024-4034.

183. Anderson K, Stott EJ, Wertz GW. Intracellular processing of the human respiratory syncytial virus fusion glycoprotein: amino acid substitutions affecting folding, transport and cleavage. J Gen Virol. 1992;73:1177-1188.

184. Rawling J, Garcia-Barreno B, Melero JA. Insertion of the two cleavage sites of the respiratory syncytial virus fusion protein in Sendai virus fusion protein leads to enhanced cell-cell fusion and a decreased dependency on the HN attachment protein for activity. J Virol. 2008;82:5986-5998.

185. Smith BJ, Lawrence MC, Colman PM. Modelling the structure of the fusion protein from human respiratory syncytial virus. Protein Eng. 2002;15:365-371.

186. Colman PM, Lawrence MC. The structural biology of type I viral membrane fusion. Nat Rev Mol Cell Biol. 2003;4:309-319.

187. Delgado MF, Coviello S, Monsalvo AC et al. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat Med. 2009;15:34-41.

188. Scott MD. Enhancemnet of oligomeric viral immunogenicity. Albany Medical College (Albany, NY). 2003;United States

189. Cook PM, Eglin RP, Easton AJ. Pathogenesis of pneumovirus infections in mice: detection of pneumonia virus of mice and human respiratory syncytial virus mRNA in lungs of infected mice by in situ hybridization. J Gen Virol. 1998;79:2411-2417.

190. Faisca P, Desmecht D. Sendai virus, the mouse parainfluenza type 1: a longstanding pathogen that remains up-to-date. Res Vet Sci. 2007;82:115-125.

191. Hirano N, Murakami T, Taguchi F, Fujiwara K, Matumoto M. Comparison of mouse hepatitis virus strains for pathogenicity in weanling mice infected by various routes. Arch Virol. 1981;70:69-73.

192. Fruijtier-Polloth C. Safety assessment on polyethylene glycols (PEGs) and their derivatives as used in cosmetic products. Toxicology. 2005;214:1-38.

193. Luciani AM, Rosi A, Matarrese P, Arancia G, Guidoni L, Viti V. Changes in cell volume and internal sodium concentration in HeLa cells during exponential growth and following lonidamine treatment. Eur J Cell Biol. 2001;80:187-195.

194. Peiris JS, de Jong MD, Guan Y. Avian influenza virus (H5N1): a threat to human health. Clin Microbiol Rev. 2007;20:243-267.

195. Skeik N, Jabr FI. Influenza viruses and the evolution of avian influenza virus H5N1. Int J Infect Dis. 2008;12:233-238.

196. Du L, He Y, Zhou Y, Liu S, Zheng BJ, Jiang S. The spike protein of SARS-CoV--a target for vaccine and therapeutic development. Nat Rev Microbiol. 2009;7:226-236.