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Polymer-Mediated Broad Spectrum Antiviral Prophylaxis: Utility in High Risk Environments

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1. Introduction

Viral infections are a significant cause of morbidity and mortality in humans throughout the world. However, modern medicine has a very limited ability to prevent viral diseases. While traditional vaccination strategies have been highly successful against a subset of viruses, the antigenic variation of viruses as well as the sheer number of viral pathogens has limited the efficacy of this approach.

This observation is exemplified by the finding that while most common respiratory infections are caused by Rhinoviruses, Coronaviruses, Adenoviruses and Orthomyxoviruses, a number of other viral families are also frequently implicated. Indeed, over 300 serologically distinct viruses are known to cause the pathology associated with the 'common cold' and 'flu'. [Spector, 1995] Furthermore, vaccinations have yet to prove effective against the single viral family (Rhinoviruses) commonly implicated in >60% of common colds; again due to the extreme antigenic variability found within even this single viral family. As a result, there are currently no broad-spectrum anti-viral prophylactics (either prescription or over-the-counter) capable of preventing or interrupting the progression of viral infections.

However, the safe, low cost, low technology, and non-toxic bioengineering of the terminally differentiated nasal pharyngeal epithelial host cells may provide a radically new antiviral prophylactic approach that gives rise to a transient, broad-spectrum, prophylaxis against virally transmitted respiratory infections (Figure 1). [McCoy & Scott, 2005, Sutton & Scott, 2010] This polymer-based technology is derivative of the polymer-based "immunocamouflage" technology of blood cells being actively developed within the Canadian Blood Services to reduce the risk of transfusion reactions and alloimmunization to donor red blood cells. [Scott *et al.*, 1997, Scott & Murad, 1998, Murad *et al.*, 1999a, Murad *et al.*, 1999b, Bradley *et al.*, 2001, Bradley *et al.*, 2002, Bradley & Scott, 2007, Rossi *et al.*, 2010b]

As schematically shown in Figure 1, the non-toxic bioengineering of the nasal cavity attenuates or prevents viral respiratory infections at the primary site of infection - the nasopharyngeal cell surface of the upper respiratory tract. Surprisingly to some, the primary mode of viral entry in respiratory diseases is via accidental inoculation of the nasal passage via contaminated hands. As demonstrated in Figure 1A, the initial inoculum (1) is typically

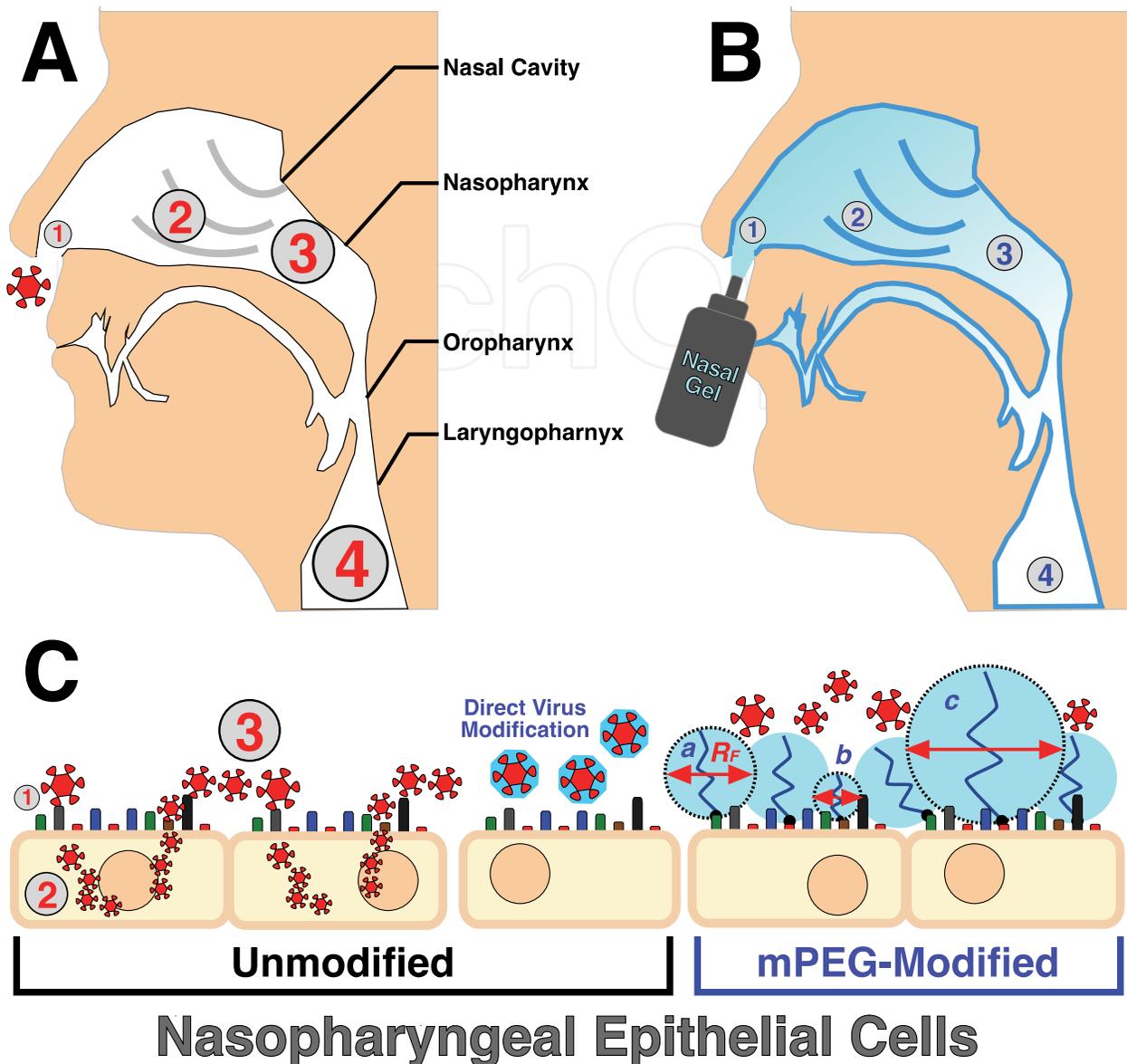


Fig. 1. Antiviral effects of nasopharyngeal immunocamouflage by activated polymer gel on disease pathogenesis. Panel A: Normal viral pathogenesis. Panel B: Effect of mPEG-Nasal Gel on viral pathogenesis. The relative efficacy of the antiviral barrier is denoted by the intensity of the blue shading. In Panels A and B, the size of the number reflects the relative viral number and disease progression: (1) Initial Inoculum; (2) invasion of adjacent cells; (3) production of progeny virus; and (4) disease progression to lower respiratory tract. Panel C: The antiviral effects of grafted polymer are shown at the epithelial cell membrane-environment interface. The efficacy of the grafted polymer is shown by the zone of protection induced by the small (*b*), medium (*a*) and large (*c*) polymers. R_F defines the radius of gyration of the grafted polymer.

small but, upon successful invasion, replicates and produces progeny virus (2) that invade adjacent cells (3) producing further progeny that may remain in the upper respiratory tract or may progress to the lower respiratory tract (4). [Winther, 2011] However, as shown in Figure 1B, the application of the activated mPEG-gel within the nasal cavity covalently modifies the terminally differentiated epithelial cells producing a physical and charge

neutralization barrier preventing viral recognition of known and unknown viral receptors. Consequent to this camouflage, the successful tissue invasion by the initial viral inoculum (1) is significantly reduced and few progeny virus (2) are produced. Subsequent replication cycles (3) are also reduced decreasing the severity or onset of disease in both the upper and lower (4) respiratory tracts. The relative efficacy of the mPEG-antiviral barrier is denoted by the intensity of the blue shading and decreases with distance from the nostril opening.

The antiviral effects of grafted polymer occurs at the epithelial cell membrane-environment interface (Figure 1C). The efficacy of the grafted polymer is dependent upon the size/topography of the viral receptor and the size (molecular weight; m.w.) of the polymer. The size of the polymer governs the Flory radii (R_F ; root mean square of end to end length of the polymer chain; radius of gyration) of the covalently bound polymers and the immunocamouflage of viral receptors. This is shown by the zone of protection induced by the small (*b*), medium (*a*) and large (*c*) polymers. Note that cell free virus can also be modified by the polymer gel resulting in an inability of the PEGylated virus to bind to its receptor. The direct PEGylation of the progeny virus further reduces the risk of clinical disease.

In contrast to traditional vaccine approaches, this novel intranasal antiviral prophylactic provides immediate, albeit transient, protection against a broad spectrum of respiratory viral pathogens. It is these pathogens that can, and do, create massive healthcare emergencies in 'at risk' populations (displaced individuals, first responders, aid workers and healthcare providers) in over-crowded refugee centers and health care facilities throughout the world. Indeed, as will be demonstrated, polymer grafting to cells results in a global, multivalent, and non-specific inhibition of viral invasion that is practical and highly suitable for rapid distribution and easily used by the target populations in a variety of environments.

2. PEGylation: Inhibition of virus-receptor recognition and binding

Our earlier studies (*e.g.*, erythrocytes, leukocytes and pancreatic islets) demonstrated that cells and tissues are readily amenable to the covalent grafting of low immunogenicity polymers to the cell membrane or tissue capsule. [Chen & Scott, 2001, Chen & Scott, 2003, Chen & Scott, 2006, Murad *et al.*, 1999a, Murad *et al.*, 1999b, Scott & Chen, 2004, Scott *et al.*, 1997] Successful immunocamouflage of cells, tissues and viruses can be accomplished by a number of polymer species such as methoxypoly(ethylene glycol) [mPEG] and hyperbranched polyglycerols [HPG]. [Scott *et al.*, 1997, Bradley *et al.*, 2002, Le & Scott, 2010, Rossi *et al.*, 2010a, Rossi *et al.*, 2010b]

Of these polymers, mPEG is the best characterized and is synthesized from poly(ethylene glycol) [HO-(CH₂CH₂O)_n-CH₂CH₂OH]. [Roberts *et al.*, 2002] The first -OH group is used to covalently attach the PEG-moiety to a linker compound that in turn is used to covalently modify cell membrane proteins. Because the second terminal -OH group of PEG confers some residual chemical reactivity, this is replaced by a -CH₃ moiety, to form activated mPEG: CH₃-(CH₂CH₂O)_n-CH₂CH₂-Linker. Multiple chemical linker groups are currently available for the grafting of mPEG to proteins. In general, the majority of these linker agents covalently react with lysine residues on membrane surface proteins (Figure 2). Consequent to this polymer grafting, our studies have demonstrated that cell charge is camouflaged and cell:cell (*e.g.*, Rouleaux formation) and receptor-ligand interactions (*e.g.*, allrecognition and antibody binding) are inhibited resulting in immunological camouflage (*i.e.*,

immunocamouflage). [Scott *et al.*, 1997, Scott & Murad, 1998, Murad *et al.*, 1999a, Murad *et al.*, 1999b, Chen & Scott, 2001, Chen & Scott, 2003, Chen & Scott, 2006, Le & Scott, 2010]

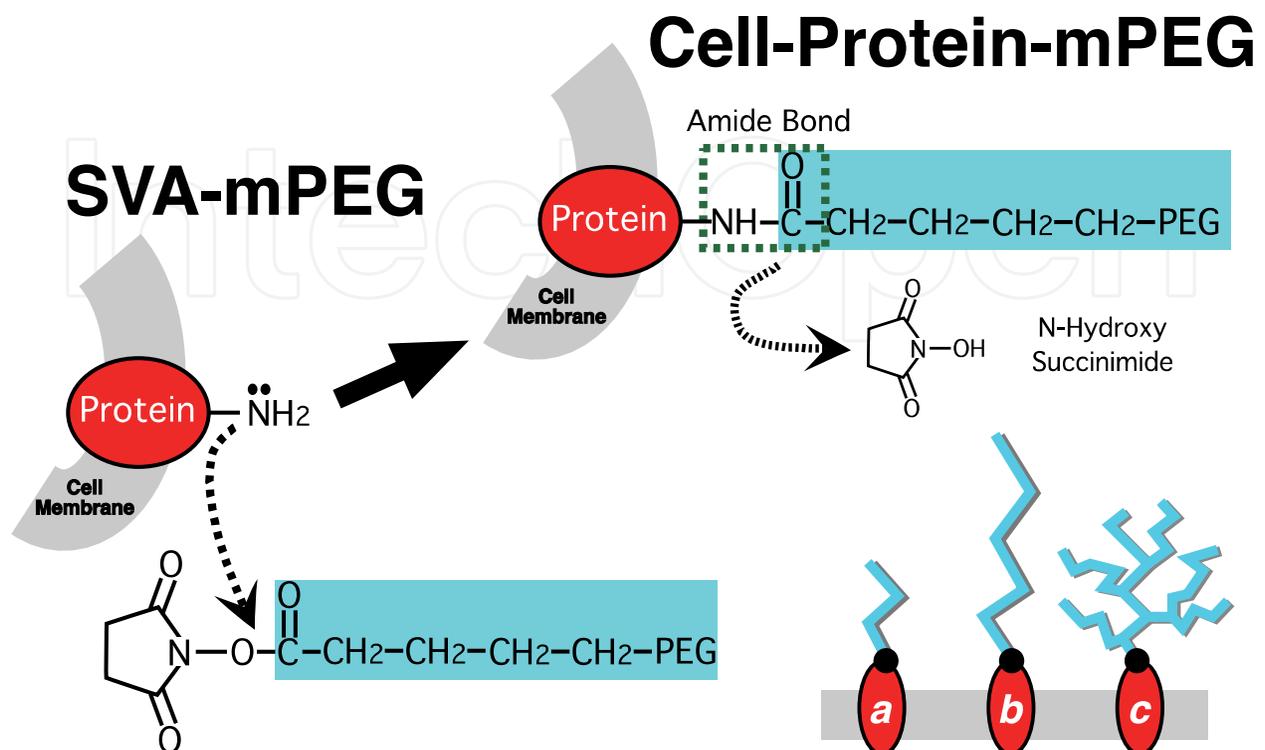


Fig. 2. Mechanism of grafting of activated mPEG to cell membrane proteins. Shown is the reaction for succinimidyl valerate methoxypoly(ethylene glycol) [SVA-mPEG]. The polymer component can be linear (*a*, *b*) of variable length (m.w.; *a*, *b* denote short and long polymers, respectively) or can be highly complex as denoted by the branched (*c*) structure. The linker chemistry is also highly variable but typically targets lysine residues on membrane proteins. Lipid anchored PEGs also exist but can negatively impact membrane fluid mechanics adversely affecting cell viability. Some work has been done with hyperbranched polyglycerols (HPG) which resemble (*c*). [Le & Scott, 2010, Rossi *et al.*, 2010b]

Importantly, viral binding to its host cell is highly analogous to receptor-ligand interactions. For viral infections to occur, viruses must bind to receptor(s) located on the cell surface (Figure 1C). Hence the immunocamouflage of either the virus or host cell would theoretically inhibit viral invasion and subsequent disease due to both charge camouflage and the steric hindrance resulting from the molecular intra-chain flexibility and rapid mobility of the heavily hydrated PEG chains (Figure 3). Moreover, the global camouflage of the cell surface effectively masks both known and unknown viral receptors resulting in a nonspecific, non-immunological, broad-spectrum antiviral effect. [McCoy & Scott, 2005, Sutton & Scott, 2010]

The biophysical basis of this antiviral effect is schematically shown in Figures 3-4. The PEG layer obscures the inherent electrical charge associated with surface proteins since the charged molecules become buried beneath the viscous, hydrated, neutral PEG layer. [Szeifer, 1997, Satulovsky *et al.*, 2000, Bradley *et al.*, 2002, Bradley & Scott, 2004, Le & Scott, 2010] As schematically shown in Figure 3, the surface of a generic cell may have a net negative charge due to the proteins and carbohydrates present on the cell surface. The

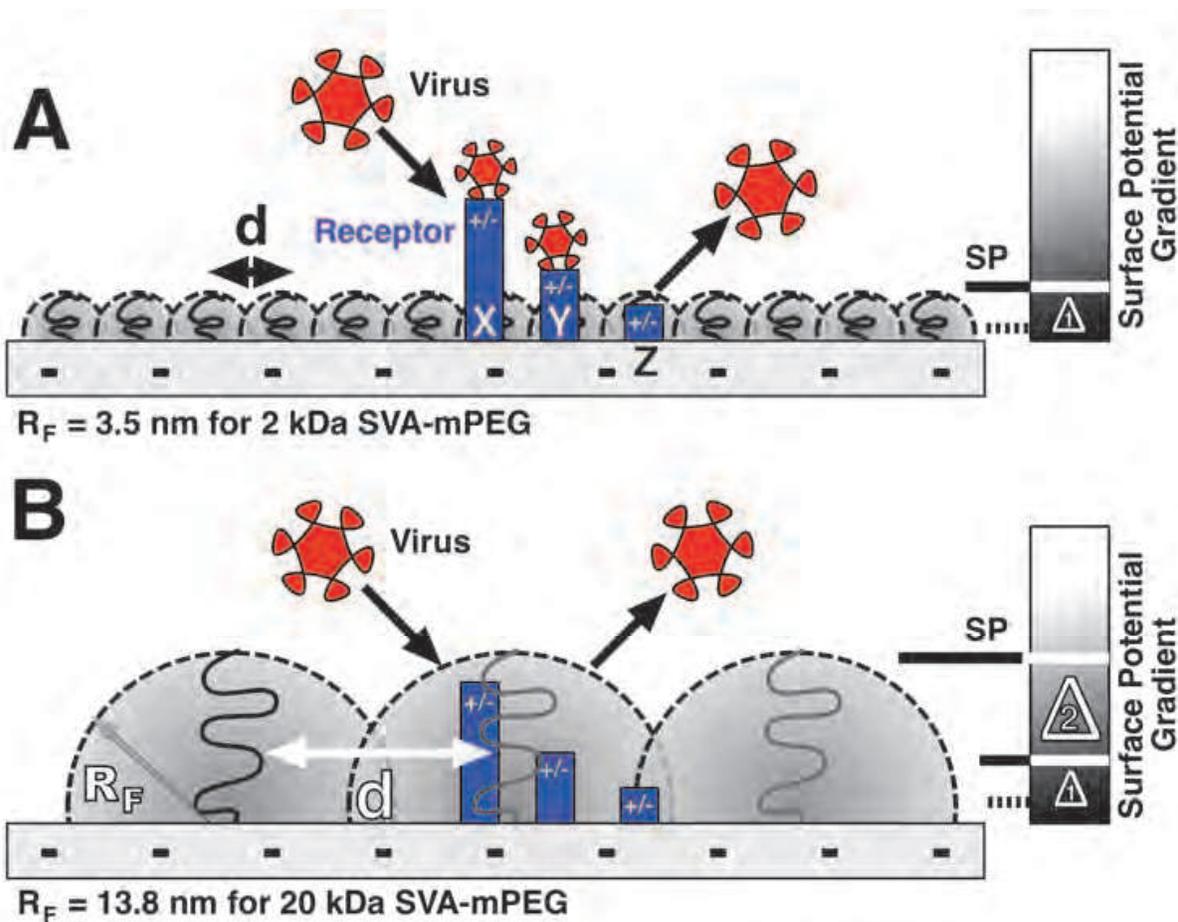


Fig. 3. Immunocamouflage of the cell surface is driven by both charge camouflage and steric interference induced by the grafted polymer. Surface charge camouflage is primarily driven by polymer-mediated extension of the shear plane (SP) of the surface towards a region of decreased surface potential (Surface Potential Gradient). In the absence of polymer, the inherent shear plane (SP) of a cell is typically located 1-3 nm above the surface. The extension of SP is proportional to the hydrodynamic thickness of the polymer layer, which in turn is governed by the Flory radii (R_F ; root mean square of end to end length of the polymer chain; radius of gyration) of the covalently bound polymers. Thus, 20 kDa polymers (large R_F ; B) provide improved charge camouflage over 2 kDa polymers (small R_F ; A). Delta (Δ) is the difference in the surface potential at the shear plane of a particle modified with the short (Δ_1) vs. the long polymer (Δ_2). The receptors X, Y and Z denote putative viral receptors extending different distances from the cell surface. Modified from [Le & Scott, 2010].

positively charged counter-ions from a bulk aqueous solution migrate and interact with the surface to neutralize the surface charge. This creates a Surface Potential Gradient, with the electric potential being the highest at the surface and decreasing with the distance away from the surface. The Shear Plane (SP) is defined as the region around the surface, where counter-ions behave as if they are physically attached to the cell and roughly approximates a neutral net charge. Polymer grafting alters the location of the shear plane relative to the membrane surface and this change is directly influenced by the size and density of the grafted polymer. As demonstrated in Figure 3A, a low molecular weight polymer (*e.g.*, 2

kDa) characterized by a small Flory radius (R_F approximating the radius of gyration of the polymer; 2 kDa mPEG has an $R_F \sim 3.5$ nm) will move the shear plane away from the surface of the cell ($\Delta 1$). [Heuberger *et al.*, 2005, Damodaran *et al.*, 2010,] However, the grafted polymer may or may not camouflage a viral receptor (X, Y, Z) depending on its topographical location relative to the SP. In contrast, a high molecular weight polymer (*e.g.*, 20 kDa; $R_F = 13.8$ nm; Figure 3B) will significantly move the SP away from the membrane ($\Delta 2$) and is more likely to result in the charge camouflage of known and unknown viral receptors (X, Y and Z). Moreover, the steric (physical) interference of the grafted polymer can also physically prevent the stable interaction of a virus with its surface receptor (Figure 3). The steric effect is maximized when chains are grafted at higher density; *i.e.*, with a small separation between the chains (d). High density grafting is difficult to achieve with polymers possessing a large R_F (*e.g.*, 20 kDa mPEG; B) as the initially bound chains sterically inhibit the approach and binding of additional polymers.

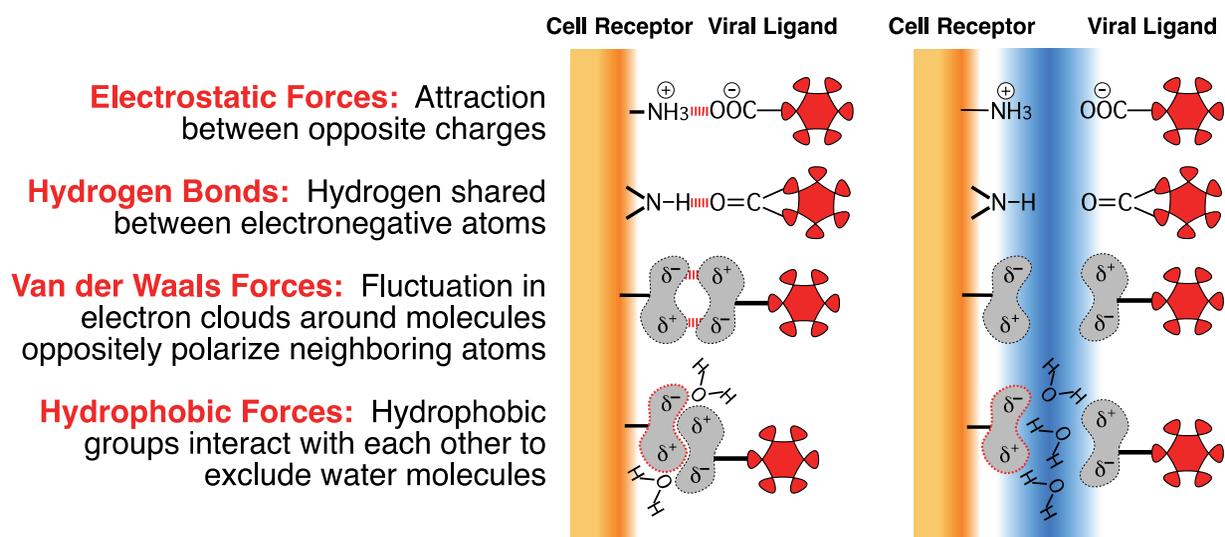


Fig. 4. Non-covalent forces mediate receptor-ligand interactions. Partial charges of electric dipoles are designated by δ . Electrostatic forces decrease as $1/r^2$, where r is the distance between the two atoms. Van der Waals forces are much weaker than electrostatic forces, because the strength of the interaction diminishes as $1/r^6$. Covalent bonds do not occur between a receptor and its ligand.

The highly malleable nature of mPEG polymers results in a broad range of possibilities of enhancing its antiviral effects via the use of both linear or branched molecules over an extraordinarily wide range of molecular weights and grafting densities (Figure 2). Of biologic importance, the absolute effects arising from both the migration of the SP within the Surface Potential Gradient and/or the steric hindrance of viral attachment need only be minor as the non-covalent forces that mediate receptor-ligand interactions are relatively weak and easily disrupted by the biophysical changes mediated by the grafted polymer (Figure 4). Thus, the bioengineering of the nasal pharyngeal epithelial cells with an mPEG nasal gel may provide significant opportunities to attenuate or block viral invasion of the initial viral inoculum, as well as any progeny, thereby reducing the both disease progression and severity.

A critical concern of this approach is the safety (acute and chronic) of the polymer. PEG is generally viewed by the US FDA as a safe compound and is widely used in food, cosmetic

and pharmaceutical formulations. Previous studies have demonstrated a lack of toxicity of PEG with polymer lengths greater than 400 Da. Human experiments dating from the late 1940's demonstrated both orally and intravenously administer PEG (*Carbowax*[®]) had no acute toxicity. [Shaffer & Critchfield, 1947, Smyth *et al.*, 1947] Indeed, intravenously administered PEGs (1 and 6 kDa) were readily excreted by the human kidney. Within 12 hours, approximately $87.2 \pm 2.3\%$ and $96.3 \pm 2.4\%$ of the 1 and 6 kDa PEG, respectively, were recovered from the urine. More recent (and ethical) studies with PEG-derivatized proteins (*e.g.*, PEG-deaminase and PEG-hemoglobin) have also demonstrated that these modified compounds (even when of xenobiotic origin) and the PEG itself have few systemic consequences and the PEG moiety is similarly excreted via the urine as found in the 1947 studies. [Veronese & Mero, 2008] PEGylated intact cells have also proven to exert no overt toxicity in a murine model. Repeated transfusions of PEGylated erythrocytes in mice have demonstrated no adverse events (*e.g.*, no hemolysis, splenomegaly, weight loss) even when >80% of their red cell mass was mPEG-RBC. [Murad *et al.*, 1999b] Moreover, there was no evidence of anti-PEG antibodies despite the massive infusion of mPEG. Indeed, after two or more decades of clinical use of PEGylated proteins, the PEG moiety has proven to be both effective in prolonging vascular retention and safe to the recipients of this therapy. [Veronese & Harris, 2002, Veronese & Mero, 2008, Veronese & Pasut, 2005]

3. Broad spectrum antiviral prophylaxis

Virally mediated respiratory diseases remain a critical problem for humans despite all our advances in pharmacology and vaccine development over the last 150 years. [Spector, 1995] This is in large part due to the sheer number of pathogens (>300 serologically distinct viruses) capable of causing the respiratory pathology associated with the 'common cold' and 'flu'. Many of these viral respiratory diseases are characterized by rapid onset and high communicability, especially when introduced into situations characterized by high population densities and poor sanitation. Indeed, in refugee centers created following natural (*e.g.*, earthquake or flooding) or man-made (*e.g.*, war, bioterrorism or incarceration) disasters, epidemic respiratory disease invariably arises within a very short time span and, along with diarrhea, is a major cause of morbidity and mortality – especially amongst the young and old. Under such emergency conditions, vaccinations (even if immunologically plausible) would be of limited utility as there would be insufficient time to adequately vaccinate the 'herd'. Rather, what is needed under these circumstances is an effective prophylactic therapy that confers significant individual, thus 'herd', protection immediately upon application. [Katriel & Stone, 2010, Van Effeltherre *et al.*, 2010, Paulke-Korinek *et al.*, 2011]

Importantly, for the intervention to be effective, it does not have to inactivate 100% of a viral threat. Rather, such prophylactic intervention must reduce the viral exposure to sub-infective levels and/or inhibit person-to-person transmission. As shown in Figure 5, all viruses have a threshold infective level. This may range from 1-2 virions per person for an extremely contagious (but not necessarily lethal) virus to several hundred or thousand viral particles in order to cause disease. The biologic objective of an activated mPEG-Gel would be to reduce the successful invasions of a respiratory virus to a level below the infectious dose necessary to cause disease. Moreover, when viewed in the context of 'herd immunity' it is also not necessary to successfully protect 100% of the at risk population to achieve significant protection of the at risk population. As shown in Figure 6, an efficacy of 50% or

75% against a theoretical respiratory virus would yield a dramatic reduction in disease progression within an at risk population.

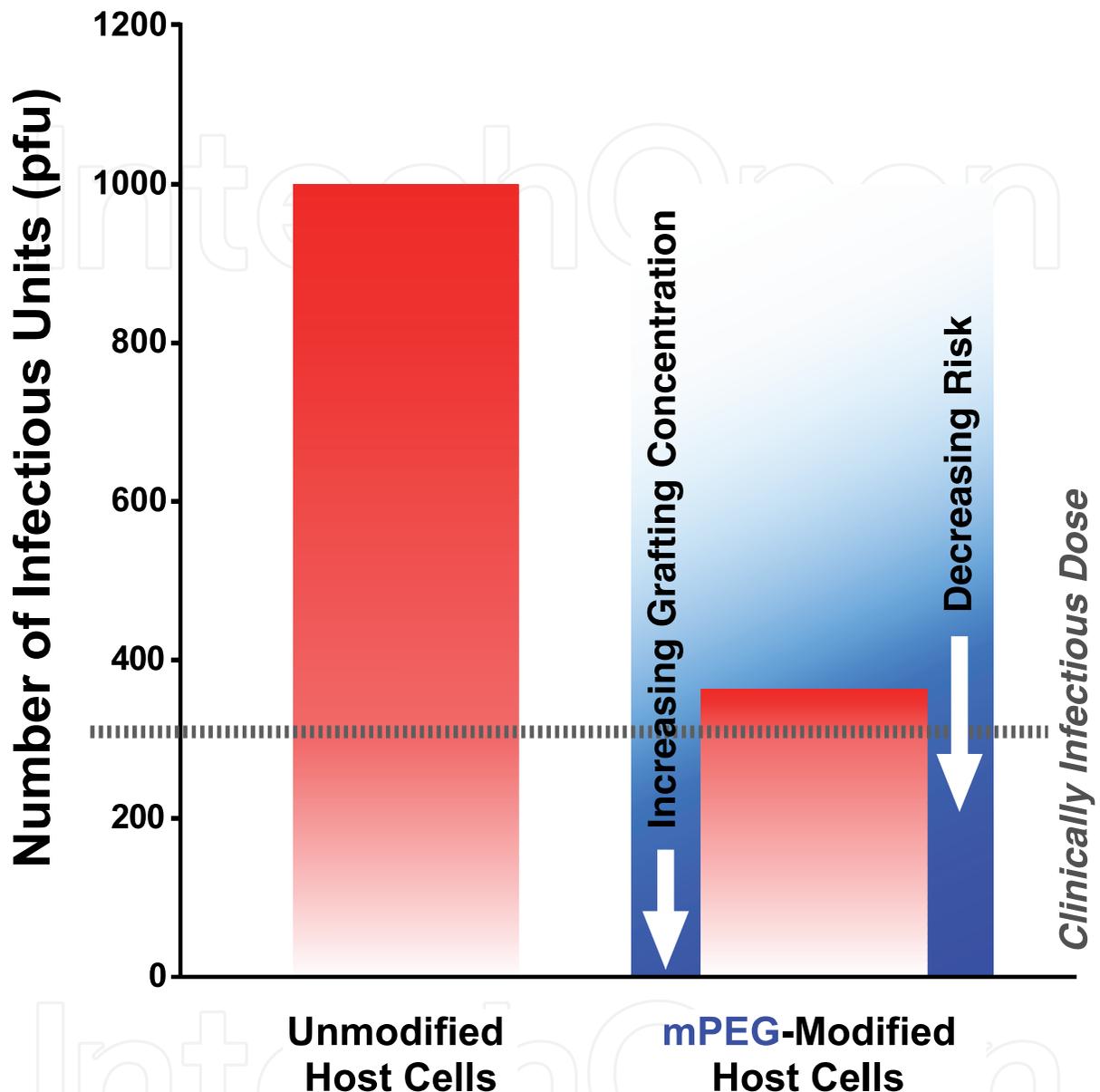


Fig. 5. The goal of mPEG-modification of either the host cells or virus is not 100% protection against viral invasion, but rather reducing the inoculation dose to below the clinically infectious dose. For example, in studies of healthy adult humans, a viral dose of 112-448 pfu (plaque forming units; a measure of viral load) of Respiratory Syncytial Virus (RSV) Long Strain A induced clinical disease in 20 of 41 volunteers. Reduction to less than this critical viral load would reduce risk of disease. [Kravetz *et al.*, 1961, Murata & Falsey, 2007]

With these key concepts in mind, we have been pursuing the functional utility and formulation of an anti-viral prophylactic polymer gel against a broad range of viruses *in vitro* (Table 1). Such protection could target either the virus or the potential target as shown in Figure 1C. Direct modification of the virus, while highly effective via this technology, is not practical for respiratory viruses (in contrast to viruses contained within blood products).

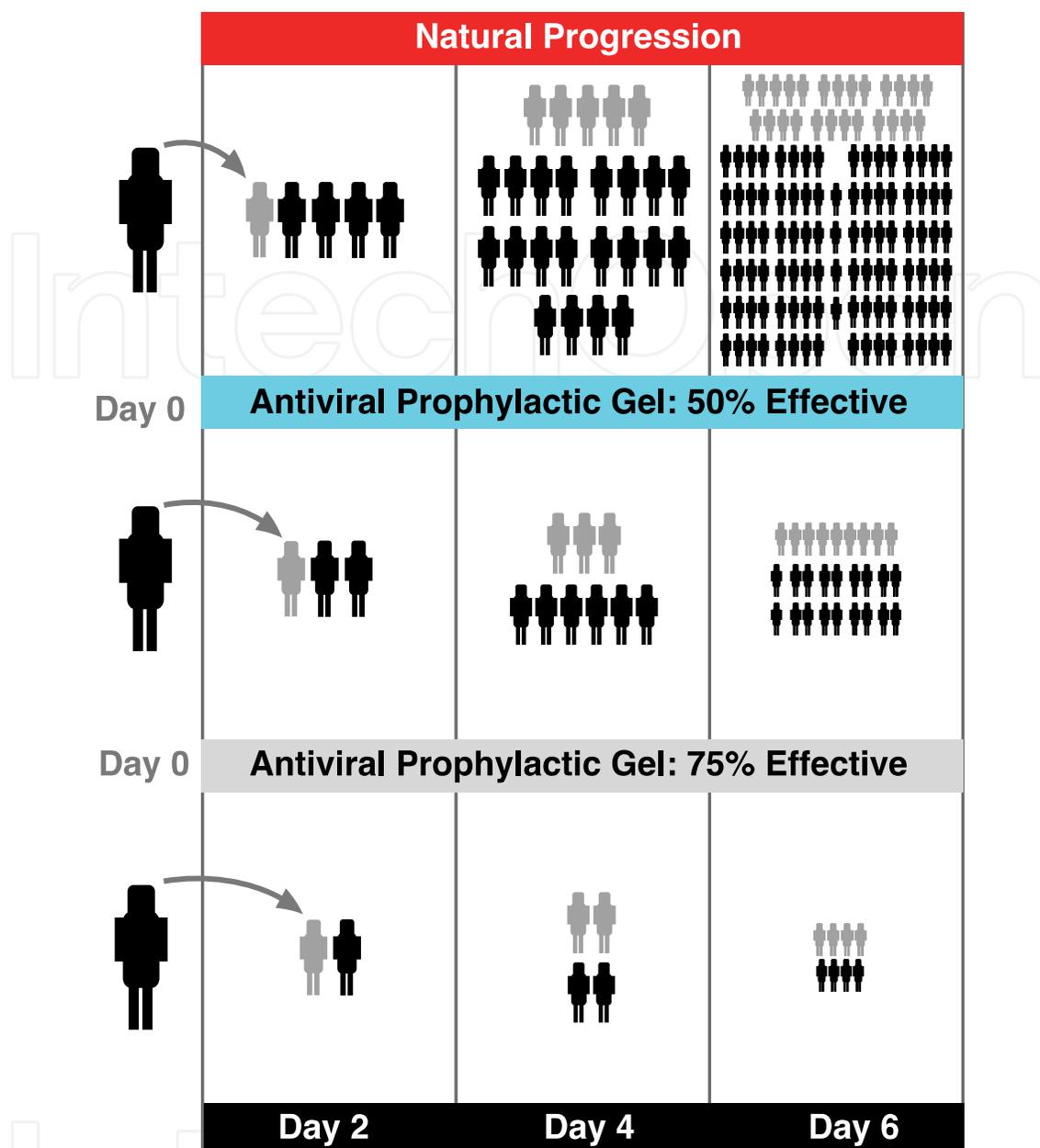


Fig. 6. Disease transmission can be dramatically reduced even if the prophylactic nasal gel is not 100% effective at the level of the individual (*Herd Immunity*). As shown, the natural progression of a hypothetical respiratory disease is such that 1 infected person transmits disease to 4 healthy individuals every 2 days. Diagrammed is the effect that a 50% or 75% effective nasal gel would have on an at risk population over 6 days. Previously infected individuals are denoted in grey. This model assumes that an individual does not die and remains contagious for a minimum of 4 days.

However, our studies suggest that the application of the activated polymer gel within the nasal cavity could prove to be a highly effective antiviral prophylactic (Figure 1B). To further explore this prophylactic approach, experimental viruses were chosen to include both enveloped and non-enveloped viruses, receptor and fusion mediated viral invasion, large and small viruses and to be representative of known human respiratory viruses (*e.g.*, Rhinoviruses, Coronaviruses, Adenoviruses).

Virus and Family	Virus Structure	Virion Size	Mode of Entry	Receptor	Receptor (nm)
Mouse Adenovirus (MAV) <i>Adenoviridae</i>	Naked Icosahedral Capsid	70-90 nm	Receptor Mediated Endocytosis	mCAR	4.6 nm
Rat Coronavirus (RCV) <i>Coronaviridae</i>	Enveloped Helical Capsid	80-160 nm	Fusion	Not identified - other family members use APN	13.5nm (APN)
Theiler's Murine Encephalomyelitis Virus (TMEV) <i>Picornaviridae</i>	Naked Icosahedral Capsid	28-30 nm	Receptor Mediated Endocytosis	Not identified - other family members use ICAM-1	18.7 nm (ICAM-1)
Respiratory Syncytial Virus (RSV) <i>Paramyxoviridae</i>	Enveloped Helical Capsid	150-300 nm	Fusion	Not Identified	Unknown
Simian Virus 40 (SV40) <i>Papovavirida</i>	Naked Icosahedral Capsid	45-55 nm	Receptor Mediated Endocytosis	MHC-1	7 nm

Murine Homologue of Coxsackie and Adenovirus Receptor (mCAR); Aminopeptidase N (APN); Intracellular Adhesion Molecule-1 (ICAM-1); and Major Histocompatibility Molecule-1 (MHC-1). SV40 is not a respiratory virus but is a well characterized experimental model suitable for experimental study. Receptor size references as follows: MHC-1 [Bjorkman *et al.*, 1987]; mCAR [He *et al.*, 2001]; APN [Hussain *et al.*, 1981]; EGFR [Mi *et al.*, 2008]; ICAM-1 [Jun *et al.*, 2001]

Table 1. Partial Listing of Experimental Viral Models Utilized in the Evaluation of the Proposed Antiviral Prophylactic Nasal Gel.

3.1 Antiviral nasal gel

To determine if an activated mPEG-gel could inhibit viral invasion and proliferation, *in vitro* studies were conducted using the viruses described in Table 1. These studies examined both direct polymer modification of the viruses themselves as well as their host (target) cells.

To determine if viruses were amenable to PEGylation by the chemistry noted in Figure 2, an analysis of envelope and capsid proteins was done. The amino acid composition and sequence of the envelope and capsid proteins suggested that all viruses had suitable targets for lysine based grafting. For example, analysis of a number of human RSV isolates demonstrate that both the F and G capsid glycoproteins are lysine rich and provide an excellent substrate for direct viral modification with mPEG. Infection assays confirmed this analysis. These experiments demonstrated that direct covalent modification of the virion by mPEG resulted in an almost complete abrogation of viral infection and proliferation [Sutton & Scott, 2010]. However some significant variability in the efficacy of small and large polymers was noted. As shown in Figure 7A, at low grafting concentrations (≤ 2 mM) small molecular weight mPEG demonstrated superiority over large polymers when modifying the virus (RSV) directly. At these low virus:mPEG ratios the improved efficacy of the 2 kDa

polymer may be partially due to the distribution of virus specific proteins within the viral envelope. On the RSV virion the G (attachment) and F (fusion) proteins form glycoprotein spikes that are 6-10 nm apart and extent 11-20 nm from the virion surface. Thus, given the distance between glycoprotein spikes on the RSV virion, grafting of 2 kDa mPEG will likely result in the direct modification of a greater number of glycoprotein spikes. Grafting of 20 kDa mPEG will likely result in fewer spikes being modified as the initially bound mPEG will exclude other polymer chains from grafting to spikes in close proximity. At higher grafting concentrations, the self-exclusion effect of grafted mPEG is partially overcome resulting in high levels of surface modification and prevention of viral binding to the host cell. Importantly, soluble mPEG (*i.e.*, unable to covalently modify the virus) had no antiviral effect. In support of these observations, our previous studies clearly demonstrated that binding of mPEG-modified RSV or SV40 to the target cells is dramatically decreased as determined by immunofluorescence staining 90 minutes post challenge at 4°C (to prevent internalization). [McCoy and Scott, 2005; Sutton and Scott, 2010]

While the utility of direct viral modification might be beneficial in blood banking environments bereft of modern viral detection methodology, this application would have little or no practical value in the prevention of respiratory disease. To this end, the *in vitro* PEGylation of the host cell monolayers were conducted. These studies have demonstrated that the selection of the polymer molecular weight and grafting concentration are important in preventing viral infection.

In contrast to Figure 7A, small chain polymers were completely ineffective when used to directly modify the RSV host cell – even at very high grafting concentrations (Figure 7B). This suggests that the unknown membrane receptor for RSV extends well away from the membrane surface and the 2 kDa polymer is unable to effectively camouflage it. However, cellular grafting of small polymers may provide partial protection against some viruses. As shown in Figure 8, low molecular weight mPEG (3.4 kDa) when grafted to host cells does provide anti-viral protection over multiple logs of viral challenge when using SV40. Even at very high levels SV40 challenge (*e.g.*, 10^{11} pfu/ml) grafted polymer ($20 > 3.4$ kDa) offered very significant protection against infection and viral proliferation as evidenced by the significant decrease in infected cells. For example, unmodified host cells infected with 10^7 pfu/mL were 27% infected, while those modified by 1.2 and 15 mM 3.4 kDa mPEG were 6% and 0.6% infected. In contrast, the 20 kDa polymer at the 1.2 and 15 mM grafting concentrations when challenged with 10^7 pfu/mL resulted in infection rates of only 2% and 0.05%, respectively. In contrast to RSV, the viral receptor for SV40 is known to be MHC-1, a molecule that extends approximately 7 nm from the cell membrane. The protection offered by the small polymer is inferior to that offered by the 20 kDa mPEG but is still significant relative to unmodified cells. As with RSV, and in all models tested, soluble mPEG (*i.e.*, unable to covalently modify the host cell surface) had no antiviral effect.

The observed relationship between polymer molecular weight and the physical size of the grafting target was consistently observed across multiple viral models. Further analysis of the relationship was examined using a latex bead – plasma adsorption model to which mPEGs of various molecular weights were grafted (Figure 9). Using small latex beads (1.2 μm), short chain polymers were more effective as noted by the decreased amount of adsorbed fluorescently labeled plasma protein. In contrast, with large 8 μm latex beads (or 8 μm RBC) large chain polymers are most effective at preventing the adsorption of the fluorescently labeled plasma. In agreement with this finding, PEGylation of viruses (0.02-200 μm) also demonstrate that short chain polymers (2 kDa) are more effective at preventing

viral invasion and infection while large chain polymers provide superior protection when grafted to host cells (e.g., RSV; Figure 7).

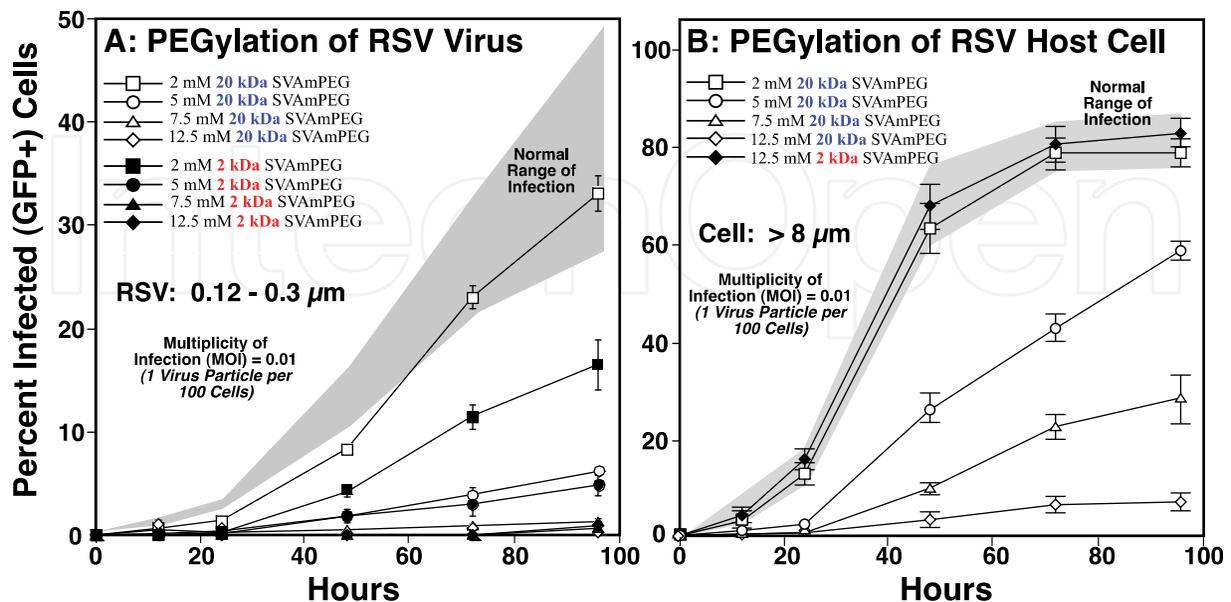


Fig. 7. PEGylation of virus (A) or host cell (B) can prevent viral invasion and proliferation. Shaded areas denote normal range of infection for control studies. An MOI of 0.01 was used for both panels. Shown are the mean \pm SD of a minimum of 3 independent experiments. Data modified from: [Sutton & Scott, 2010]

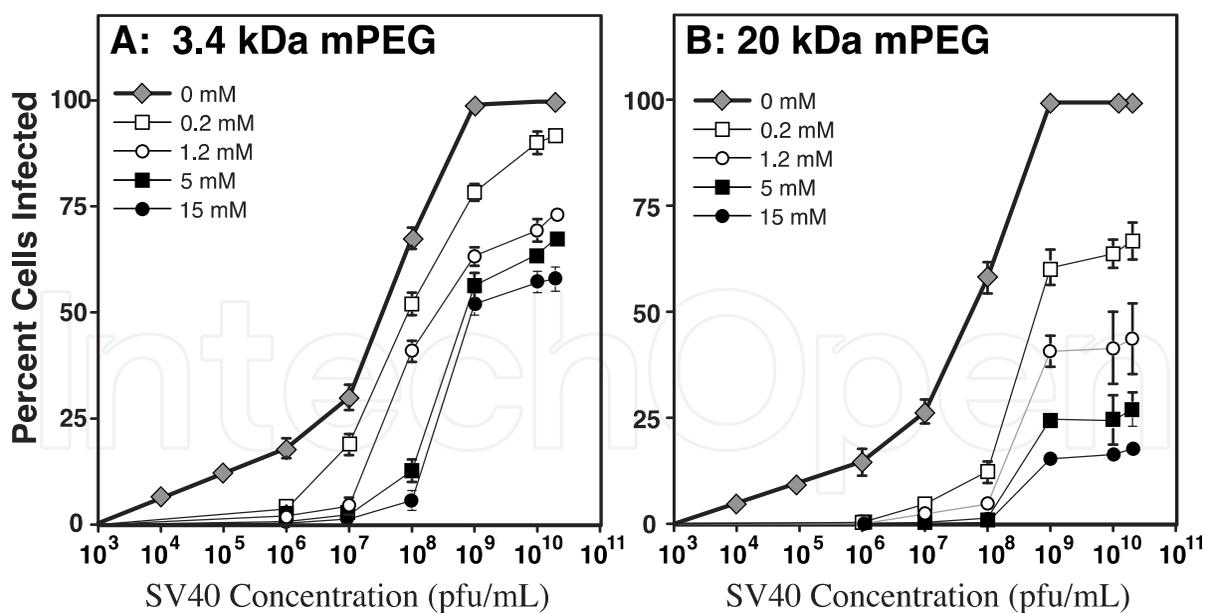


Fig. 8. In contrast to RSV, infection by other viruses is partially blocked by the grafting of short chain polymers to the host cells. CV-1 cells were modified with the indicated concentrations of activated mPEGs of 3.4 (A) or 20 (B) kDa and challenged over a broad range of viral titers. Percent infected cells was determined at 72 hours via T antigen staining. The mean \pm standard deviation of 3 independent experiments are shown. Data modified from: [McCoy & Scott, 2005]

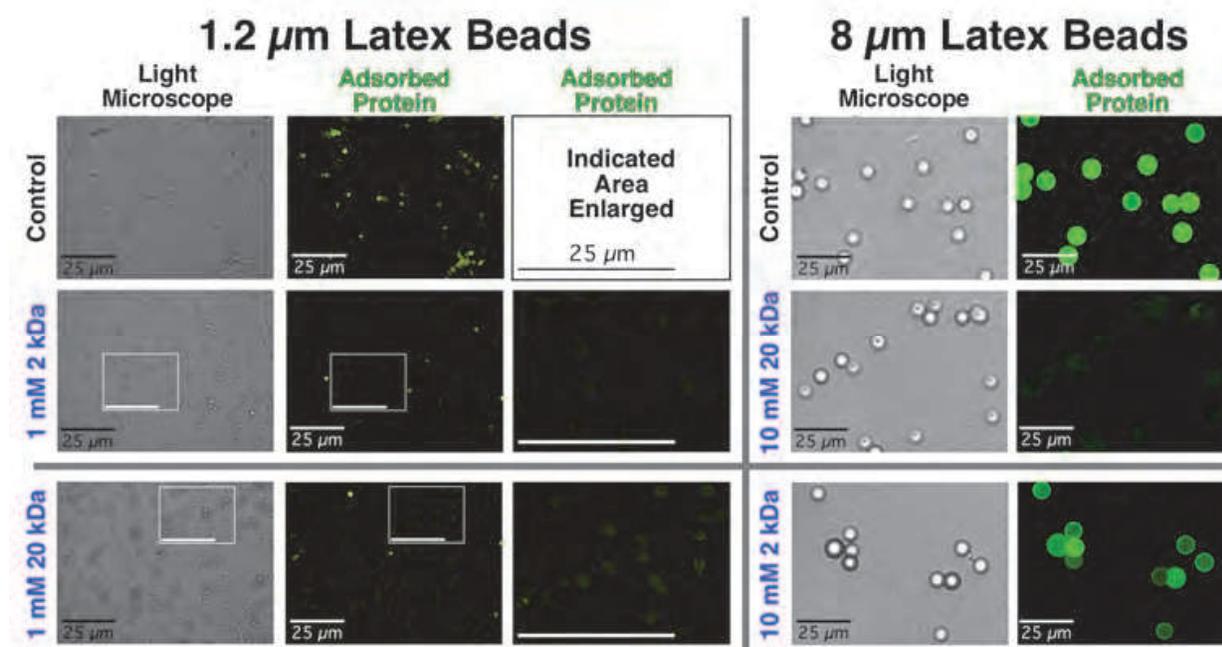


Fig. 9. Paradoxically, the size of the PEGylation target influences which polymer size most effectively imparts immunocamouflage. Modified from Le and Scott (2010). [Le & Scott, 2010]

To determine if a nasal gel would adequately cover the tissue of interest, *in vivo* studies were conducted. To assess the area of coverage, a polymer gel formulation was labeled with India Ink and administered intranasally (150 μ l per nostril) in a Guinea Pig model of Respiratory Syncytial Virus (RSV) infection. These studies demonstrated full coverage of the nasal cavity and even significant protection to the level of the lung (Figure 10).

Indeed, studies using a pigmented nasal gel demonstrated that a gel can provide protection to both the upper and lower respiratory tract in a Guinea Pig model of respiratory Syncytial Virus (RSV). Dissection of the Guinea Pig nasal cavity demonstrated uniform staining of the cavity surface. Moreover, as shown in Figure 10, application of a PEG-based gel containing India Ink resulted in significant protection even within the lung tissue.

To date, we have demonstrated that the covalent grafting of mPEG to virus itself or to the host/target cell results in a highly effective broad-spectrum antiviral prophylaxis. Consequent to the immunocamouflage of the host cell, viral entry and propagation by members the *Picornaviridae*, *Adenoviridae*, *Cornaviradae*, *Pneumovirus* (all representative of common human respiratory viruses) families as well as members of the *Papovaviridae* and *Hepesviridae* families are virtually ($\geq 90\%$) blocked even at high viral titers. Furthermore, this antiviral prophylaxis is effective against: 1) enveloped and non-enveloped viruses; 2) receptor-mediated and fusiogenic viruses; 3) small and large viruses; 4) DNA and RNA viruses; and 5) viruses with known and unknown viral receptors. The mechanism of this protection is biophysical (not immunological) in nature and dependent upon charge camouflage and steric hindrance induced by the grafted polymer. Importantly, due to the basic biophysical nature of this antiviral effect, it is not prone to biological adaptation (*i.e.*, sequence mutations obviating the efficacy of antibodies) by the virus.

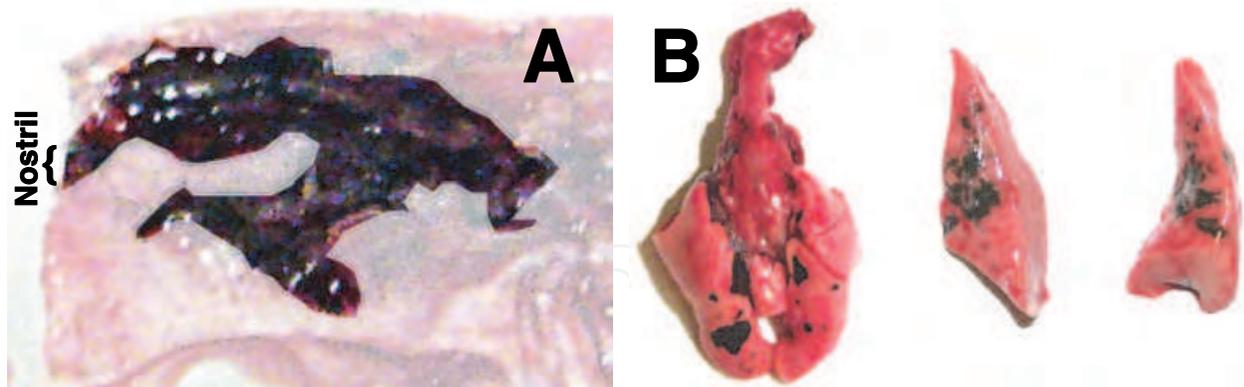


Fig. 10. Penetration of the nasal gel within the nasal cavity (A) and to the lower respiratory tract (B) was examined within a Guinea Pig model of Respiratory Syncytial Virus (RSV) infection. Shown are the nasal cavity and whole lung and sagittal sections of the lung lobes one hour after intranasal administration of 300 μ l (150 per nostril) of India ink labelled polymer gel. The non-sinus tissues is partially masked. Note the distribution of the ink, indicative of the successful delivery of the nasal polymer to the nasal cavity and regions of the lung.

3.2 Alternative preventative or therapeutics approaches

Current methods to prevent respiratory viral disease range from homeopathic drug treatments (*e.g.*, mega doses of vitamin C, oral zinc and Echinacea) to pharmacological interventions such as anti-viral drugs and vaccination. Many of these methods have been proven ineffective (vitamin C) or are not always practical or effective (*i.e.*, mass vaccination) in preventing disease. [Block & Mead, 2003, Yale & Liu, 2004]

Potential pharmacological means of preventing viral infections are to block either viral entry, or proliferation, within the host cell. However again there are no United States FDA-approved *broad spectrum* prophylactic therapies or anti-viral treatments capable of preventing either of these events. Most approved pharmacological antiviral agents exhibit high specificity. Among the best described prescription antiviral agent is Amantadine (FDA approved in 1966), which is used to prevent the uncoating of influenza A. Within cells, Amantadine specifically inhibits viral uncoating by blocking the activity of the proton channel of the influenza A M2 protein preventing the endosomal pH change required for uncoating to occur. [Kandel & Hartshorn, 2001] However, Amantadine has only a brief window of efficacy and is highly specific to influenza A (*i.e.*, completely ineffective against other influenza strains much less different viral families). Most recently the U.S. Center for Disease Control (CDC) recommends against the use of Amantadine due to viral resistance. In its place, the CDC has recommended the use of Tamiflu® (Oseltamivir; FDA approved 1999) to prevent or treat influenza A and B infections. Oseltamivir is a neuraminidase inhibitor that is specific to influenza and blocks the exit of the progeny influenza viruses from the host cell, thus limiting viral replication and disease progression. However, the efficacy of Tamiflu® has recently been reassessed ([Jefferson *et al.*, 2009]) and found to offer only modest prophylaxis when administered within 24 hours of viral infection. Other prescription antiviral drugs demonstrate similar limited or absent efficacy against other respiratory viral families (Rhinoviruses, Coronaviruses, and Adenoviruses) associated with cold or flu causation.

Thus, patients typically turn to over-the-counter (OTC) cough and cold remedies which focus on the relief of symptoms associated with the common cold. Currently in North America alone an estimated \$3-6 billion (US) is spent on symptom relief. A few OTC compounds do attempt to prevent or, after infection, attenuate viral infection. These commercial products include compounds such as ZICAM Nasal Gel (active ingredient: zinc gluconate) and ColdFX (active ingredient: proprietary natural extract containing poly-furanosyl-pyranosyl-saccharides in a concentration of greater than 80%). [Hirt *et al.*, 2000, McElhaney *et al.*, 2006] For drug efficacy, ZICAM must be applied intranasally every 2-4 hours. However, Zicam's proven mode of action is highly specific and, in laboratory studies, only prevents infection by those rhinoviruses that use ICAM1 as the viral receptor. [Bella & Rossmann, 1999] Hence, it is completely ineffective against the majority of cold viruses that do not use ICAM1 (*e.g.*, Coronaviruses and Adenoviruses). More recently, in 2009 ZICAM's original intranasal formulation was removed from the market due to adverse side effects. [Lim *et al.*, 2009] ColdFX's mode of action and viral range of efficacy are not as well documented, though clinical studies do suggest variable degrees of efficacy in small population studies. Studies cited by ColdFX suggest that the natural ingredients stimulate the immune system (*e.g.*, activate macrophages, increase natural killer (NK) cell numbers) to both prevent initial viral infection and to more efficiently kill off virally infected cells to prevent viral proliferation. [Wang *et al.*, 2004, Miller *et al.*, 2009] However, direct experimental evidence for these purported actions is limited.

Other experimental approaches have also been investigated but are also highly specific to single viral strains and/or families. For example, two other experimental methods of preventing rhinoviral infection have been studied which, like ZICAM, targets the rhinovirus:ICAM-1 interaction. These are the intranasal application of soluble Intracellular Adhesion Molecule (sICAM) or anti-ICAM-1 antibodies (rhinovirus receptor murine monoclonal antibody; RRMA). Like ZICAM, these experimental drugs require intranasal application every 2-4 hours. [Marlin *et al.*, 1990, Huguenel *et al.*, 1997, Turner *et al.*, 1999] Both of these drugs do demonstrate some *in vitro* and *in vivo* (murine) efficacy against rhinoviruses. Randomized human trials with sICAM demonstrated that intranasal administration of sICAM 7 hours prior to or 12 hour post rhinovirus challenge resulted in a relative reduction in cold symptoms but very weak prevention (92% of the placebo-treated subjects and 85% of the sICAM-treated subjects became infected). [Turner *et al.*, 1999] Similarly, human trials with RRMA demonstrated no reduction in rhinovirus infection or disease severity. [Hayden *et al.*, 1988, Sperber & Hayden, 1989] Furthermore, repeated administration of the mouse derived RRMA would also lead to rapid immunization against the drug. Importantly, unlike the described mPEG nasal gel, both sICAM and RRMA are highly specific to only those Rhinoviruses that utilize ICAM-1 as the viral receptor. For example, when sICAM was tested against herpes simplex virus type 1 (HSV-1), coxsackie virus B1 or poliovirus no observable protective effect was noted. [Marlin *et al.*, 1990]

Thus, the antiviral prophylactic polymer nasal gel illustrated here represents the only broad spectrum antiviral prophylactic agent described to date. Application of the activated nasal gel is uncomplicated and requires a minimal (~3-5 minutes) application time to effectively camouflage known and unknown viral receptors. After application, the individual then simply "blows their nose" into a tissue to remove any residual carrier fluid. Current data suggests that a single daily application provides maximal protection and retains significant efficacy for up to 60 hours. A brief comparison of a cross sample of the above approaches with our proposed antiviral nasal gel is shown in Table 2A/B.

A. Anti-Viral Activity Against Respiratory Viruses

Viral Family	mPEG Nasal Gel	RRMA ^a	Soluble ICAM	Zicam	Traditional Vaccination
<i>Adenoviridae</i>	+	-	-	-	+/-
<i>Coronaviridae</i>	+	-	-	-	+/-
<i>Herpesviridae</i>	+	-	-	-	+/-
<i>Papovaviridae</i>	+	-	-	-	+/-
<i>Picornaviridae</i>	+	+*/-	+*/-	+*/-	-
<i>Retoviridae</i>	+	-	-	-	+/-
<i>Paramyxoviridae</i>	+	-	-	-	+/-

B. Pharmacological Traits

	Gel	RRMA ^a	Soluble ICAM	Zicam	Traditional Vaccination
Non-Specific Protection	Yes	No	No	No	No
Non-Immunogenic	Yes	?	?	+	+/-
Lack of Inherent Resistance	Yes	?	?	+/-	+/-
Longevity of Protection by a Single Application	24-48 Hr	2-4 Hr*	2-4 Hr*	2-4 Hr*	**

^a Anti-ICAM Antibody; +/- Highly Strain Specific; * Only against Rhinoviruses utilizing ICAM-1 as viral receptor; ** Protection if generated, can last weeks to years.

Table 2. Comparison of the antiviral (A) and pharmacological (B) traits of the described mPEG-Nasal Gel to other experimental and commercial antiviral agents.

3.3 Non-respiratory antiviral applications

Are there other applications for an antiviral prophylactic gel? Humans are beset by a host of viral pathogens and common to all of these pathogens is the need to gain entry into its target cell in order to proliferate. Not all viruses or modes of entry (*e.g.*, *i.v.* drug use) would be amendable to an antiviral gel. However, the use of an antiviral gel in preventing or reducing the risk of an initial infection by the Human Immunodeficiency Virus (HIV) could be envisioned. [Piguet & Steinman, 2007, Cutler & Justman, 2008, Boily *et al.*, 2009, Garg *et al.*, 2010]

In both male-female and male-male transmission, the HIV virus typically enters the host via Langerhans cells, a resident epidermal dendritic cell, dermal dendritic cells, and/or resident CD4⁺ lymphocytes all of which are present within the mucosal and sub-epithelial tissues of the anogenital region (Figure 11A). Viral entry is gained primarily via two receptors located on the surface of the cell: CD195 (CCR5) and CD184 (CXCR4). [Piguet & Steinman, 2007] If dendritic (antigen presenting) cells capture the virus, it is either endocytosed or can be transferred directly to CD4⁺ CD195⁺ T lymphocyte via an infection synapse. Proliferation of the virus within T cells is followed by infection of additional lymphocytes via either CD184 or CD195. Importantly, all of these events are analogous to cellular invasion by respiratory

viruses and amenable to disruption by grafted polymer. In a model analogous to spermicide or lubricant, an activated polymer gel could be applied either vaginally or anally prior to at-risk behavior creating an antiviral barrier on the epithelial cell surfaces of the mucosal tissue (Figure 11B) and preventing viral binding via the biophysical mechanisms already described. As shown in Figure 12, proof-of-concept studies clearly demonstrate that grafting of mPEG to CD184 and CD195 positive cells inhibits recognition of these markers by high affinity antibodies.

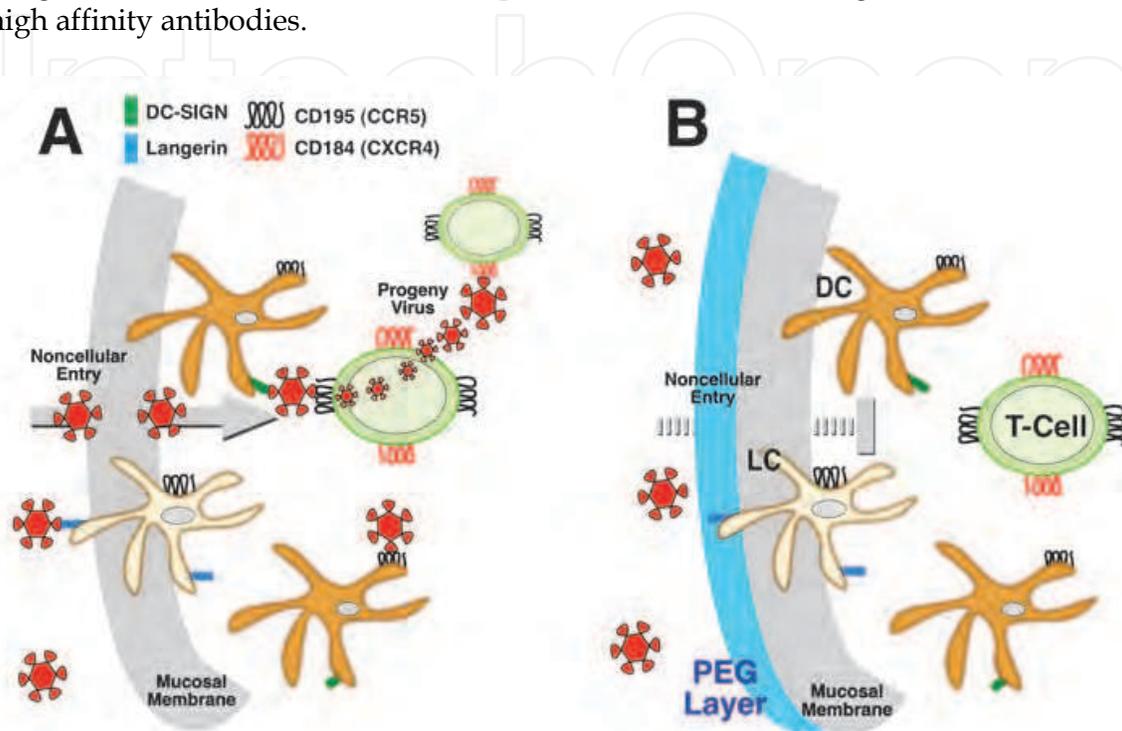


Fig. 11. Immunocamouflage of HIV receptors by activated mPEG antiviral gel. Panel A) Normal mode of HIV invasion; Panel B) Blockade of HIV binding by activated mPEG gel. Langerhan Dendritic Cells (LC); Dermal Dendritic Cells (DC); CD4+ T Lymphocyte (T-Cell). Langerin, a lectin present on LCs is a primary attachment point for HIV. A different lectin (DC-SIGN) is present on other DC and serves as a receptor for HIV. Noncellular entry may occur via epithelial cell injury (e.g., microabrasions).

Because the risk of HIV transmission per single interaction is relatively low (estimated to be 0.04% for female-to-male; 0.08% for male-to-female; and 1.7% for receptive anal intercourse; Boily *et al.*, 2009), the application of an effective activated mPEG-based mucosally applied antiviral gel would further reduce the risk of transmission. Moreover, PEG itself is a lubricant and would reduce the risk of traumatic tissue injury and microabrasions – known risk factors in HIV transmission. The lubricant effect of the mPEG-gel would be of particular importance with regards to anal transmission as the anal epithelium ranges from a multi- to single-layer epithelial lining prone to tearing (in contrast to the vagina which is a multicellular stratified squamous epithelium and resistant to traumatic injury). This simple anatomical difference in large part underlies the differential transmission risk associated with anal intercourse. Finally, the immunocamouflage of the infected dendritic cells and macrophages would also make the cells less prone to activation by other pathogens due to the camouflage of key surface receptors. In the absence of activation, HIV proliferation within infected cells is greatly diminished decreasing viral shedding.

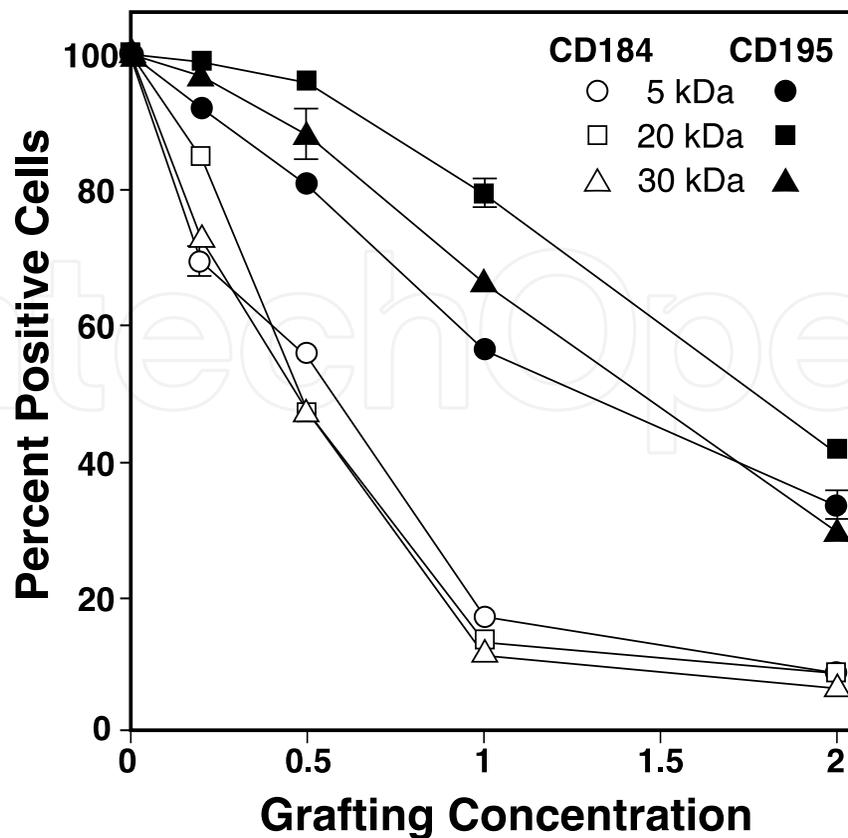


Fig. 12. Immunocamouflage of HIV receptors by activated mPEG antiviral gel. As shown, PEGylation of dendritic cells and lymphocytes results in the immunocamouflage of CD184 and CD195 as detected by high affinity monoclonal antibodies to the CD determinants. The efficacy of the immunocamouflage is concentration dependent.

Indeed, there are several topical microbiocidal gels in clinical trials that aim to prevent against HIV infection either non-specifically (surfactants and acidifying agents) or specifically (viral entry and reverse transcriptase inhibitors). Some surfactants, such as nonoxynol-9, experimentally were shown to result in viral disruption but also exerted toxicity to host cells. Further clinical trials of nonoxynol-9 in HIV endemic areas actually demonstrated increased risk of HIV infection with its use. [Rustomjee *et al.*, 1999, Van Damme *et al.*, 2002, Herold *et al.*, 2011] These studies suggested that the surfactant-induced mucosal injury appeared to mimic the presence of microabrasions already known to increase HIV transmission. Other surfactants such as sodium lauryl sulfate (invisible condom) disrupt enveloped and non-enveloped viruses and are associated with decreased (relative to nonoxynol-9) mucosal toxicity. [Howett & Kuhl, 2005, Mbopi-Keou *et al.*, 2010] In contrast to surfactants, the mPEG antiviral gel is of low toxicity to cells while simultaneously providing significant camouflage of the mucosal surface thereby preventing or decreasing the initial viral interaction with resident dendritic cells and T lymphocytes.

4. Conclusion

The safe, low cost, low technology, non-toxic, and transient bioengineering of the terminally differentiated nasal pharyngeal epithelial host cells may provide a radically new antiviral prophylactic approach. Moreover, this approach may be applicable to other non-respiratory

viruses in which a polymer gel can be applied. This antiviral protection arises from the ability of the grafted polymer to directly impede cellular invasion and subsequent proliferation thus abrogating the disease process at its initial stage. Moreover, this technology is effective against: 1) enveloped and non-enveloped viruses; 2) receptor-mediated and fusigenic viruses; 3) small and large viruses; 4) DNA and RNA viruses; and 5) viruses with known and unknown viral receptors.

The envisioned use of this prophylactic technology is via a simple intranasal gel application in at risk individuals in acute, high transmissibility, environments (*e.g.*, nosocomial spread, refugee centers, bioterrorism, airplanes, or with visiting grandchildren). For example, within refugee centers, epidemiological analyses demonstrate that, alongside malnutrition and diarrhea, acute respiratory infections are a major cause of morbidity and mortality (*e.g.*, Figure 6). Under identical circumstances, traditional vaccines, even if they were available, would not be able to prevent widespread dissemination of disease while this novel intranasal anti-viral prophylactic provides immediate, albeit transient, protection against a broad spectrum of respiratory viral pathogens.

Application of the activated nasal gel is surprisingly uncomplicated and requires a minimal (~3-5 minutes) application time to the host (*e.g.*, nasopharyngeal) cells but effectively camouflages known and unknown viral receptors thus protecting against a broad spectrum of viral pathogens. Of note, nasal epithelial cells are terminally differentiated and slough off after about 4 days and are biologically *outside* the body resulting in minimal systemic exposure to the mPEG polymer. The polymer itself has an extremely safe toxicity profile and is commonly used in current pharmaceuticals (most often as a stabilizer or binding agent), cosmetics and food preparations. After application, the individual then simply “blows their nose” into a tissue to remove any residual carrier fluid. Furthermore, preliminary data suggests that a single daily application provides maximal protection and even retains significant efficacy for up to 60 hours.

Respiratory pathogens can, and do, create massive healthcare emergencies in at risk populations. The development of this inexpensive, easy to use intranasal antiviral gel would be of significant health and economic benefit to both the at risk individual and to governmental and health agencies addressing respiratory disease crises. Importantly, the term ‘*at risk*’ is relative and may range from environmental or political upheavals to more mundane environments such as air travel and visiting grandchildren.

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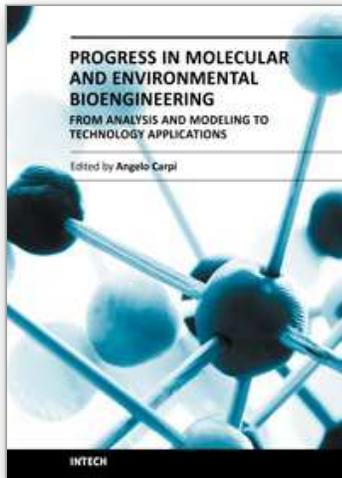
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This book provides an example of the successful and rapid expansion of bioengineering within the world of the science. It includes a core of studies on bioengineering technology applications so important that their progress is expected to improve both human health and ecosystem. These studies provide an important update on technology and achievements in molecular and cellular engineering as well as in the relatively new field of environmental bioengineering. The book will hopefully attract the interest of not only the bioengineers, researchers or professionals, but also of everyone who appreciates life and environmental sciences.

How to reference

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