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The effect of grafted methoxypoly(ethylene glycol) chain length on the inhibition of respiratory syncytial virus (RSV) infection and proliferation

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ABSTRACT

Respiratory syncytial virus (RSV) is a significant cause of morbidity in humans. To date, no effective treatments exist and current prophylactic therapy access is limited and is only ~50% effective. To attenuate the risk of RSV infection, we hypothesized that bioengineering of either the virus particle or host cell via the covalent grafting of methoxypoly(ethylene glycol) [mPEG] would prevent infection. To this end, the anti-viral effects of grafting concentration, linker chemistry and polymer length on RSV infection was assessed. For viral modification, short chain polymers (2 kDa) were significantly more effective than long chain (20 kDa) polymers. In contrast, modification of host cells with small polymers provided no (~0%) 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. Importantly, both viral and host cell PEGylation strategies were able to provide near complete protection against RSV infection of both non-polarized and polarized cells. In conclusion, mPEG-modification of either RSV or the host cell is a highly effective prophylactic strategy for preventing viral infection.

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1. Introduction

Respiratory syncytial virus (RSV) is a contagious virus belonging to the family Paramyxoviridae [1]. RSV infects nearly all children in the first two years of life [2] and is the major cause of bronchiolitis and pneumonia in this population [3]. As a result, RSV infection is the leading cause of infant hospitalization in the Western world [4]. To date, limited treatments exist for severe RSV infection and no safe effective RSV vaccine has been developed. Indeed, current prophylactic treatment is limited to the monthly administration of humanized mouse anti-RSV antibody preparations (Synagis) that reduce the incidence of severe disease by ~50% [5,6]. However, due to expense, these preparations are only administered to infants at high risk for severe RSV infection [7].

Previously we have demonstrated that covalent grafting of methoxypoly(ethylene glycol)[mPEG] to the surface of red blood

cells prevents immunological recognition (termed “immunoca-mouflage”) without altering cell lysis, morphology, and the hemoglobin oxidation state [8–10]. Furthermore, grafting of mPEG to T cells prevents allorecognition and T cell activation demonstrating that mPEG grafting inhibits receptor–ligand interaction of cell surface proteins [11,12]. Analogous to these allogenic receptor–ligand interactions, we have shown that direct mPEG-modification of simian virus 40 (SV40) prevents host cell infection [13]. Furthermore, mPEG-modification of host cell surface proteins protected cells from viral challenge with Theiler’s murine encephalomyelitis virus (TMEV), mouse adenovirus (MAV), rat coronavirus (RCV), and SV40 [13]. Thus, we sought to evaluate mPEG grafting directly to either RSV or its host cell as the initial phase in developing a prophylactic anti-RSV nasal spray [13–15].

In this study, we have investigated whether mPEG-modification of the RSV virion or the host cell can be an effective strategy for preventing viral infection. Both strategies were evaluated over a broad range of viral infective doses and in both polarized and non-polarized host cells. Finally, the effects of particle size (virus versus mammalian cell) relative to the anti-viral efficacy of the short or long chain grafted polymer was investigated.

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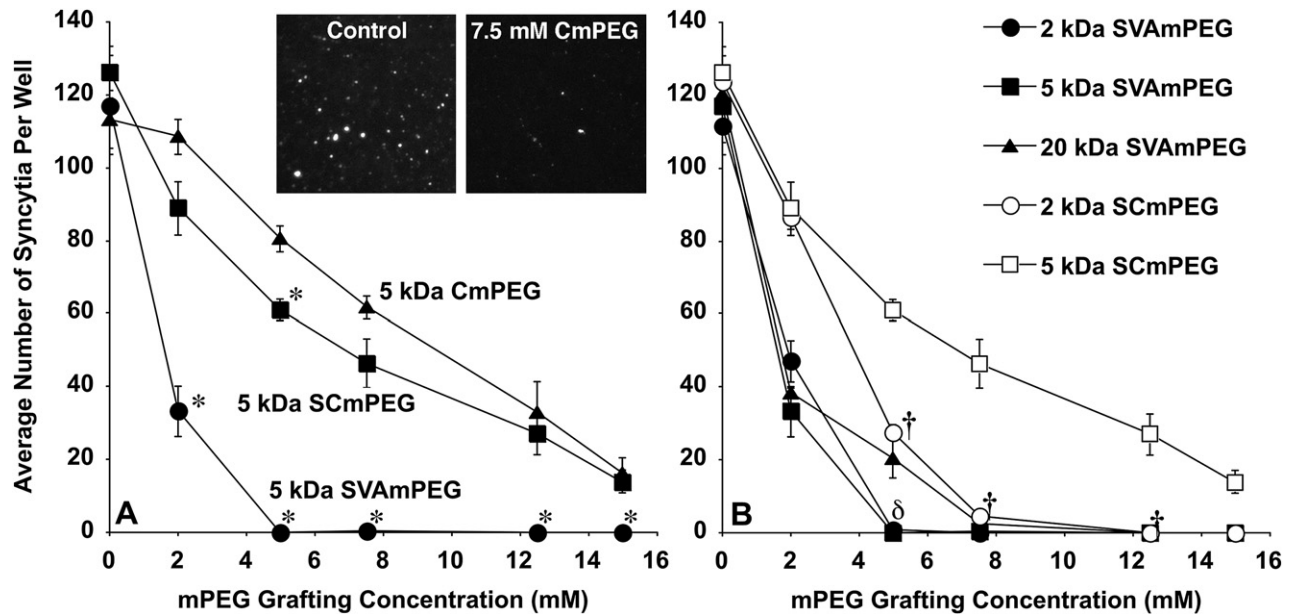


Fig. 1. mPEG-modification of RSV prevented syncytia formation in a dose-dependent manner. As shown, short chain polymers and SVA-linker chemistry exhibited enhanced efficacy at lower grafting concentrations. At higher grafting concentrations short and long chain polymers demonstrated equivalent protection. (A) Anti-viral effect of RSV modification using three different linker chemistries coupled to a 5 kDa polymer. mPEG-modification of RSV prevented infection (i.e., syncytia formation) in a dose-dependent manner with SVAmPEG showing enhanced protection relative to SC- and CmPEG. Insert: binding of mPEG-RSV to target cells was visualized by immunofluorescence staining 90 min post-challenge at 4 °C (to prevent internalization) with an MOI of 0.5. (B) Comparison of 2 and 5 kDa SCmPEG, and 2, 5 and 20 kDa SVAmPEG-modification of Long Strain A RSV. As discussed, SVAmPEG was superior to SCmPEG in preventing infection by polymer modified virus. Short chain polymers, especially using the SCmPEG, were statistically superior to long chain polymers. Values shown are the number (mean \pm standard error) of syncytia per well at five days post-infection of a minimum of three experiments performed in duplicate. Significance: *significantly different from all other mPEG species at the equivalent grafting concentration ($p < 0.026$); \dagger significantly different from the 5 kDa SCmPEG at the equivalent grafting concentration of 2 kDa SCmPEG ($p < 0.012$); and (δ) significantly different from 20 kDa SVAmPEG ($p < 0.025$).

2. Materials and methods

2.1. Cell lines and virus strains

Vero, HeLa, Madin–Darby canine kidney (MDCK) cells, and RSV Long Strain A were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Recombinant green fluorescent protein expressing RSV (rgRSV), clone 224 derived from the RSV A2 strain [16] was obtained from Dr. M.E. Peeples (Nationwide Children's Hospital, University of Ohio, Columbus, Ohio) and Dr. P.L. Collins (National Institute of Allergy and Infectious Diseases, Bethesda, Maryland).

Vero and MDCK cells were grown in minimal essential media (MEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, MEM vitamin solution (1 \times), L-glutamine (2 mM), sodium pyruvate (1 \times), 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) supplemented with 10% FBS, GlutaMAX-I (2 mM), HEPES buffer (1 mM), penicillin (0.292 mg/mL), and streptomycin (100 μ g/mL) (Invitrogen). RSV Long Strain A and rgRSV were grown in Vero and HeLa cells, respectively, and viral stocks were prepared by collection of the supernatant from lysed-RSV infected cells. For all experiments and culture of rgRSV, media lacking phenol red was used.

2.2. mPEG species

Cyanuric chloride activated mPEG (CmPEG; 5 kDa) was purchased from Sigma–Aldrich (Sigma–Aldrich, Oakville, ON, Canada). Succinimidyl carbonate mPEG (SCmPEG; 2 and 5 kDa), succinimidyl valerate mPEG (SVAmPEG; 2, 5 and 20 kDa, and fluorescein-labeled 20 kDa) were purchased from Laysan Bio Incorporated (Arab, AL, USA).

2.3. mPEG-derivatization of RSV

For direct virus modification, RSV Long Strain A or rgRSV were diluted in 2% FBS-supplemented MEM or DMEM media (pH 7.8), respectively. Sample volumes were adjusted to yield final mPEG grafting concentrations of 0, 2, 5, 7.5, 12.5, and 15 mM at the indicated viral multiplicity of infections (MOI). Concentrated polymer solutions were prepared immediately prior to use (to prevent hydrolysis of the linker chemistry) and the derivatization reaction was done at room temperature for 30 min. The mPEG-modified virus solutions were subsequently overlaid on unmodified host cells for 90 min at 37 °C with gentle rocking every 15 min unless otherwise noted.

2.4. mPEG-derivatization of host cells

For host cell membrane surface modification, cells were grown to 90–100% confluence. Prior to derivatization, the cells were rinsed once with PBS and subsequently overlaid with media (pH 7.8) containing freshly prepared activated mPEG at the indicated grafting concentrations. Cells were modified at constant volume to surface area ratio of 0.2 mL/cm² and were incubated with the activated mPEG solution for 30 min at room temperature. Following modification, cells were rinsed with 2% FBS-supplemented media and then challenged with unmodified virus.

2.5. Viral plaque assays

Vero cells were seeded in 24-well tissue culture plates (Becton Dickinson) and incubated until 90–100% confluent. The cell monolayer were then overlaid with 0.4 mL (~125 plaque forming units; pfu) of control or mPEG-modified RSV Long Strain A for 90 min at 37 °C. The virus solutions were removed and the monolayers were overlaid with a pre-warmed 1% methylcellulose (Sigma) in 2% FBS-supplemented MEM media. Culture plates were incubated for 5 days at 37 °C prior to syncytia counting. A minimum of three independent experiments, each with duplicate samples, were conducted.

2.6. Virus dose studies

For both virus and host cell modification studies, rgRSV was used to challenge non-polarized HeLa cells. HeLa cells were seeded in 96-well plates (Becton Dickinson) and incubated overnight until 90–100% confluent. Virus and host cell polymer (2 and 20 kDa) grafting were conducted as described at mPEG concentrations of 0, 2, 5, 7.5, 12.5, and 15 mM. Viral challenge studies of either the control and modified virus (90 min overlay) or the control and modified host (HeLa) cells (30 minutes overlay) were done at MOI of 0, 0.01, 0.05, 0.1 and 0.5. Samples were incubated for 12, 24, 48, 72, and 96 h post-challenge. At the indicated time points, cells were harvested and analyzed by flow cytometry as described previously [16]. A minimum of five thousand events were acquired and analyses were conducted by gating on sham-infected cells (MOI = 0).

2.7. rgRSV infection of polarized cells

To evaluate the efficacy of mPEG grafting (both virus and target cell) in a polarized cell model, MDCK cells were used in conjunction with rgRSV. MDCK cells were grown to confluence in 96-well plates, and then challenged with either mPEG-

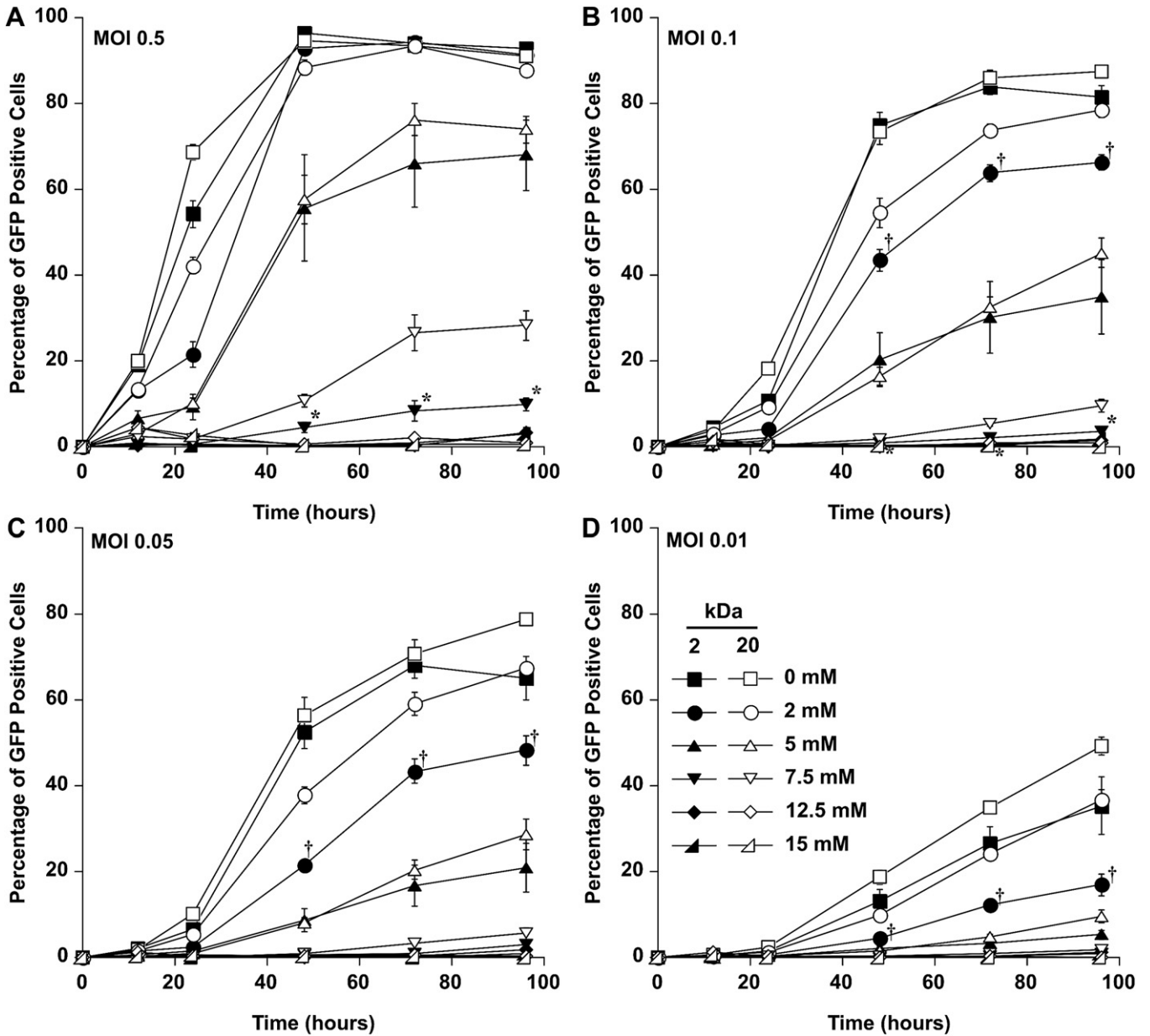


Fig. 2. Polymer grafting to RSV resulted in a significant reduction in viral infection across a broad range of MOI. Shown are modification of rgRSV with 2 kDa (open symbols) or 20 kDa (solid symbols) SVAmPEG at at MOI of 0.5, 0.1, 0.05, and 0.01 (A, B, C, and D, respectively). Modification of rgRSV with either 2 or 20 kDa SVAmPEG resulted in an mPEG dose-dependent decrease in the percentage of GFP positive (rgRSV infected) cells. At low to moderate grafting concentrations, the 2 kDa mPEG showed a trend towards enhanced protection relative to the 20 kDa polymer. Values expressed are mean \pm SE of two separate experiments performed in quadruplicate. Significance: *significantly different from the 20 kDa polymer length at the 7.5 mM grafting concentration ($p < 0.04$); and †significantly different from the 20 kDa polymer length at the 2 mM grafting concentration ($p < 0.04$).

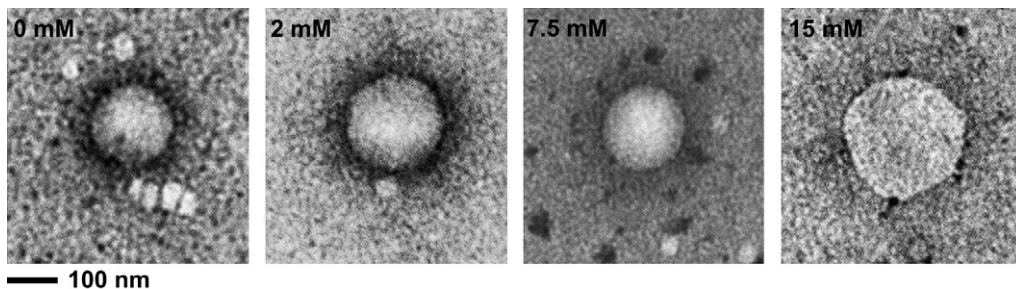


Fig. 3. mPEG-modification of RSV did not disrupt normal virion morphology as evidenced by electron microscopy. Shown are representative transmission electron microscope (TEM) images of rgRSV virions modified at grafting concentrations of 0, 2, 7.5, and 15 mM SVAmPEG (2 kDa). rgRSV images were taken at 100,000 \times magnification.

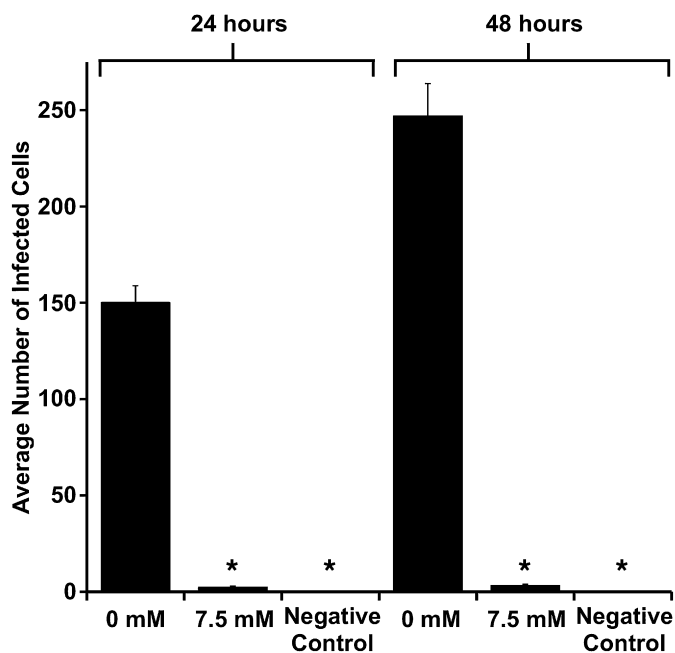


Fig. 4. mPEG-modification of rgRSV dramatically decreased infection and proliferation in polarized MDCK cells. Polarized MDCK cells were challenged with 2 kDa SVAmPEG-modified RSV at a grafting concentration of 7.5 mM. The virus concentration was equal to MOI 8 due to the decreased susceptibility of MDCK cells to RSV infection. Values shown are mean \pm SE for two separate experiments performed in quadruplicate. Significance: *significantly different from the 0 mM condition ($p < 0.001$).

modified virus or subjected to pegylation prior to challenge with unmodified virus. For virus modification, rgRSV was PEGylated with 2 kDa SVAmPEG, while for host cell modification, MDCK cells were PEGylated using 20 kDa SVAmPEG. To overcome the decreased susceptibility of this cell line to RSV infection [17], an MOI of 8 was used and cells were challenged for 12 h at 37 °C and then washed and overlaid with 2% FBS-MEM. The plates were then incubated for 24 or 48 h and GFP expressing cells were visualized and counted using a fluorescent light microscope (Olympus).

2.8. Transmission electron microscopy

Transmission electron microscopy (TEM) of negatively stained rgRSV was performed using a Hitachi H7600 microscope (Hitachi High Technologies America Inc., Pleasanton, CA, USA). At a virus concentration equivalent to an MOI of 0.5, rgRSV was modified with 2 kDa SVAmPEG in 7.5 mL of 2% FBS-media at pH 7.8. 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) and virus was concentrated according to the manufacturers instructions. Subsequently, mPEG-modified virus was diluted, and stained as described previously [18,19]. Individual virus particles were then visualized and images were captured at 100,000 \times magnification.

2.9. Statistical analyses

For all analyses, SPSS v12 statistical software (Statistical Products and Services Solutions, Chicago, IL, USA) was used with a two-tailed p -value of < 0.05 considered statistically significant. Data from the virus dose 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. 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, student- t tests were performed.

3. Results

To determine if mPEG-modification of RSV inhibited viral infection, we initially performed plaque assays with 5 kDa CmPEG. As shown in Fig. 1A, CmPEG-modification of RSV decreased the number of syncytia in a dose-dependent manner. To determine if linker chemistry influenced the anti-viral efficacy, we investigated

the effects of 5 kDa SC- and SVAmPEG on syncytia formation (Fig. 1A). As noted, both SCmPEG and SVAmPEG resulted in a dose-dependent decrease in the number of syncytia. However, relative to Cm- and SCmPEG, SVAmPEG resulted in significantly greater inhibition of RSV infection at all grafting concentrations ($p < 0.026$) with the exception of 15 mM where all linker chemistries yielded nearly complete protection. Importantly, as demonstrated by the insert in Fig. 1A, binding of mPEG-modified RSV to the target cells is dramatically decreased as determined by immunofluorescence staining 90 min post-challenge at 4 °C (to prevent internalization).

To evaluate the effect of polymer length on RSV inactivation, we compared 2 and 5 kDa SCmPEG, and 2, 5 and 20 kDa SVAmPEG (Fig. 1B). With all polymer lengths and linker chemistries examined, grafting of mPEG resulted in a dose-dependent decrease in the number of syncytia. Comparing the two polymer lengths containing the SC linker chemistry, the 2 kDa mPEG was superior to the 5 kDa chain at grafting concentrations of 5, 7.5, and 12.5 mM ($p < 0.012$). Importantly, at both 7.5, 12.5 and 15 mM grafting concentrations, the 2 kDa SCmPEG virtually abolished RSV infection while the 5 kDa SCmPEG did not. The SVA-linker chemistry provided superior protection at the lower grafting concentrations at all three molecular weights (2, 5, and 20 kDa; Fig. 1B). While some statistical advantage ($p < 0.025$) was seen with the 2 and 5 kDa polymers over the 20 kDa SVAmPEG at the 5 mM grafting concentration, this was not thought to be of biologic significance. Importantly, at high mPEG grafting concentrations (≥ 12.5 mM) all SVAmPEG species completely prevented RSV infection and propagation.

To determine the efficacy of this anti-viral prophylactic approach, the effect of polymer grafting to RSV was assessed over a broad range of MOI. Virus MOI ranging from 0.5 to 0.01 were modified with 2 or 20 kDa SVAmPEG for HeLa cell challenge (Fig. 2). As shown, at the very high MOI of 0.5, low grafting concentrations of SVAmPEG (2 mM) of either the 2 or 20 kDa polymers were ineffective at preventing viral invasion. However, as grafting concentration increased (≥ 5 mM) a dose-dependent decrease in viral infection was noted with a near complete abrogation of infection at ≥ 12.5 mM. Interestingly, at the 7.5 mM grafting concentration at an MOI of 0.5, the 2 kDa mPEG polymer was significantly ($p < 0.006$) more effective at preventing viral infection than the 20 kDa chain (Fig. 2A). As the MOI decreased, the relative dose-dependent efficacy of mPEG grafting increased due to a greater polymer to virus ratio (i.e., more chains per viral particle). An interesting finding was that, as a general trend, RSV modification with short chain polymers (2 kDa) provided improved anti-viral efficacy over long (20 kDa) chain polymers, especially at lower grafting concentrations (e.g., MOI 0.05 and 0.01 at 2 mM grafting concentration; $p < 0.007$). At higher RSV grafting concentrations (≥ 12.5 mM), both polymer lengths yielded nearly complete protection against viral infection at all MOI. Across all MOI examined, a grafting concentration of 7.5 mM produced the most dramatic decrease in infection per unit (mM) of mPEG.

To demonstrate that viral PEGylation does not simply disrupt the virus particles, TEM studies were conducted. As shown in Fig. 3, TEM qualitative studies of unmodified (0 mM) and mPEG-modified (2, 7.5 and 15 mM) rgRSV showed no differences in the number of virus particles (data not shown) or RSV virion structure at any of the tested grafting concentrations. In agreement with previous reports, both unmodified and modified virus ranged in size from 150 to 300 nm [20]. Taken together, these findings indicate that mPEG grafting did not dramatically disrupt or alter the size of the RSV virion but did effectively prevent viral infection.

Because RSV specifically infects polarized airway epithelial cells in vivo, and Vero and HeLa cells are unpolarized, we evaluated mPEG-modification of RSV in the polarized MDCK cell line. As

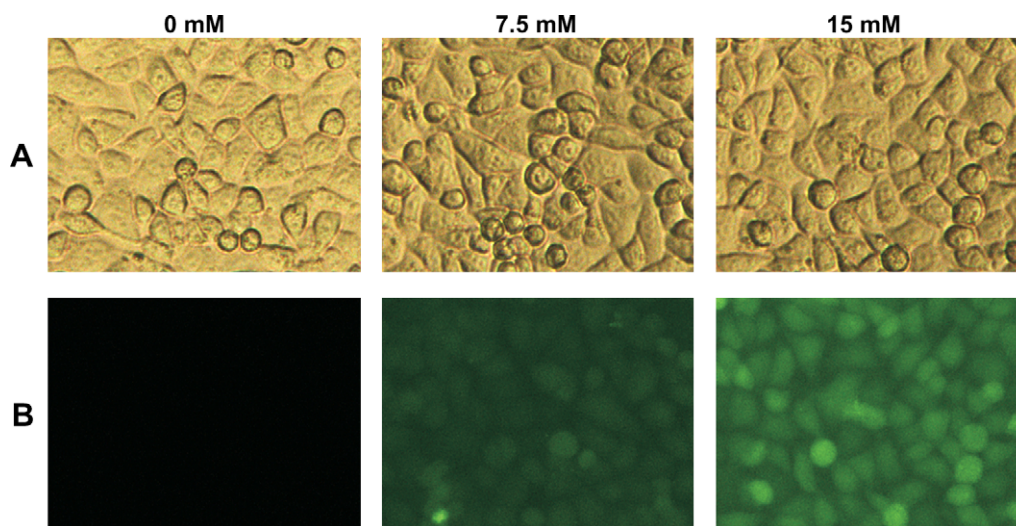


Fig. 5. Fluorescein-labeled mPEG-modification of HeLa cells demonstrated both the uniform grafting of the polymer to the host cells and the normal morphology of the modified cells. Cells were modified with a mixture of 5% Fluorescein-labeled mPEG and 95% unlabeled 20 kDa SVAmPEG. Light and the corresponding fluorescent images of control and modified cells (7.5 and 15 mM grafting concentration) are shown at 20 \times magnification. Images are representative samples of quadruplicate experiments.

shown in Fig. 4, mPEG-modification (7.5 mM) of rgRSV significantly decreased the number of infected cells at both 24 and 48 h post-infection ($p < 0.001$). Furthermore, from 24 to 48 h, the number of infected cells in the 0 mM control increased dramatically, while no significant increase was noted in cells challenged with mPEG-modified virus. This finding suggests that the virions that managed to infect the cells did not generate significant numbers of progeny to overcome the initial reduction in infection.

While direct PEGylation of virus particles is possible under certain circumstances (e.g., blood derived products such as red cells, platelets and plasma), it is less biologically useful for respiratory viruses such as RSV. However, induction of an anti-viral barrier by the direct PEGylation of the target cells (e.g., nasal epithelial cell) of the virus would be of significant clinical utility. To determine if activated mPEG resulted in uniform derivatization of cell monolayers, HeLa cells were PEGylated using a mixture of 5% fluorescein-labeled 20 kDa mPEG and 95% unlabeled 20 kDa mPEG and examined by light and fluorescent microscopy.

As shown in Fig. 5, light microscopy demonstrated that control and mPEG-modified cells exhibited normal morphology. Fluorescent microscopy of the mPEG-modified cells demonstrated uniform fluorescence and increased with mPEG grafting concentration. This result indicates that the polymer grafted to the cell surface in a dose-dependent fashion. Given that the cells were modified with only 5% fluorescein-labeled mPEG it is likely that the cell surface is significantly modified. Furthermore, trypan blue staining of the cell layer demonstrated $\geq 98\%$ viability 48 h post-modification. Flow cytometric studies of propidium iodide stained cells also demonstrated no significant decrease from initial viability levels over 24 h incubation post-modification at any mPEG (maximum of 15 mM) grafting concentration (data not shown).

To assess the anti-viral efficacy of host cell modification, cells were differentially modified with 2 or 20 kDa SVAmPEG and challenged with unmodified rgRSV at MOI of 0, 0.01, 0.05, 0.1, and 0.5. As shown in Fig. 6, the 2 kDa polymer was ineffective at any MOI or grafting concentration. Thus, while directly grafting the 2 kDa polymer to RSV effectively inhibited infection, the same polymer when grafted to host cells had no anti-viral effect suggesting that it was incapable of camouflaging the viral receptor. In dramatic contrast, the 20 kDa polymer demonstrated significant anti-viral prophylaxis at all MOI and in a mPEG dose-dependent manner. As

noted, even at a very high and non-biologic MOI (0.5; Fig. 6A) very significant reductions in infection are noted at 20 kDa polymer grafting concentrations of ≥ 5 mM. At a more biologically relevant MOI of 0.01 (Fig. 6D), host cell grafting concentrations of ≥ 5 mM of the 20 kDa polymer yielded almost complete protection against viral infection.

Importantly, at all MOI, grafting concentrations of ≥ 5 mM of the 20 kDa mPEG to the target cells resulted in a significant decrease in viral infection relative to the positive control (0 mM) over the entire 96 h incubation period. This suggests that mPEG grafting to the host cells both dramatically inhibited the initial viral invasion and also prevented the spread of infection by progeny virus. This is important, as it indicates that the grafted mPEG barrier remains intact over the 96 h incubation time. Moreover, at the lower MOI (i.e., 0.01) which approximated a natural inoculation dose, our findings indicated that mPEG-modification of host cells would reduce or completely prevent infection in vivo.

As previously discussed, RSV preferentially infects polarized epithelial cells in vivo. To confirm our findings in non-polarized cells, the anti-viral effect of membrane grafting of the 20 kDa SVAmPEG (7.5 mM) to polarized MDCK cells was assessed. As demonstrated in Fig. 7, a significant reduction ($p < 0.001$) in rgRSV infection was observed. Indeed, relative to control (0 mM) infections, reductions of 80% and 95% were seen at 24 and 48 h, respectively. Furthermore, while the number of infected cells doubled between 24 and 48 h in the 0 mM control, there was no increase in the level of infection with the 7.5 mM grafting concentration. Indeed, a slight decrease in the number of infected cells was observed – most likely due to death of the initially infected cells. This finding, as with non-polarized HeLa cells, demonstrated that the mPEG anti-viral barrier on the host cell surface was stable and able to inhibit infection by any progeny virus, thereby preventing secondary infections. These findings suggest that the mPEG-modification of the nasal epithelial cells may be capable of producing a highly effective anti-viral barrier.

4. Discussion

Based on our previous viral studies [13], we evaluated mPEG-modification of RSV as a means of inhibiting RSV infection. Using both plaque assays and rgRSV infection of HeLa cells, we showed

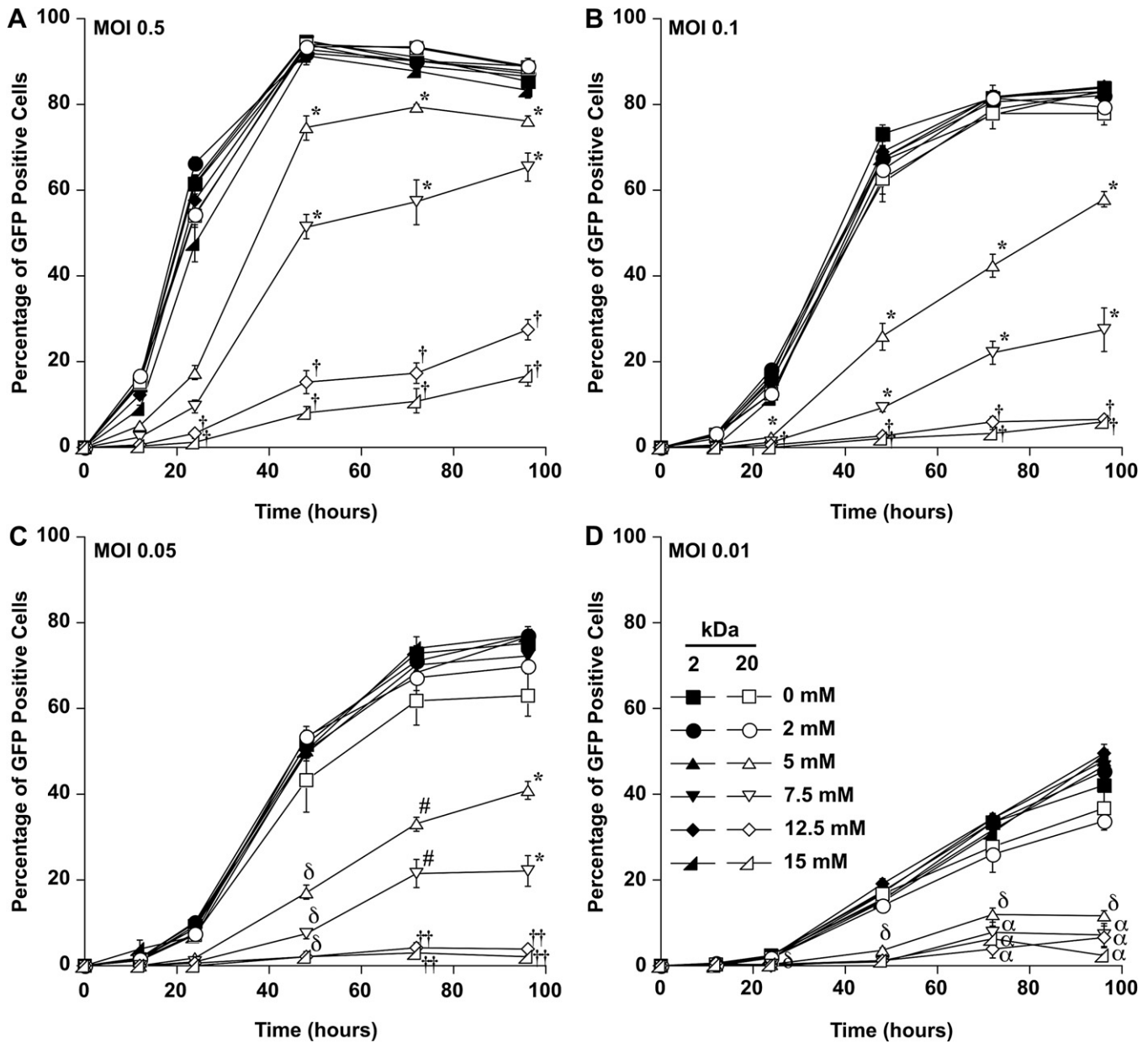


Fig. 6. In contrast to RSV modification, SVAmPEG-grafting to target (HeLa) cells demonstrated that only the 20 kDa polymer conferred protection against viral infection. Surprisingly, modification of the host cells with 2 kDa SVAmPEG did not provide any significant protection against rgRSV. Control and modified HeLa cells were challenged with rgRSV at MOI of 0.5, 0.1, 0.05, and 0.01 (A, B, C, and D, respectively). The anti-RSV effect of the 20 kDa SVAmPEG was grafting concentration dependent with near maximal protection occurring at ≥ 7.5 mM SVAmPEG. Values expressed are mean \pm SE of two independent experiments performed in quadruplicate. Significance: *significantly different from 2 kDa mPEG and from all other mPEG concentrations at the same polymer length ($p < 0.038$); †significantly different from 2 kDa mPEG ($p < 0.001$), and 0, 2, 5, and 7.5 mM at the same polymer length ($p < 0.035$); (δ) significantly different from all 2 kDa mPEG grafting concentrations, and 0 and 2 mM 20 kDa SVAmPEG ($p < 0.03$); (α) significantly different from all 2 kDa mPEG grafting concentrations, and 0, 2, and 5 mM 20 kDa SVAmPEG ($p < 0.003$); #significantly different from all 2 kDa mPEG grafting concentrations, and 0, 2, 12.5, and 15 mM 20 kDa SVAmPEG ($p < 0.01$); and ††significantly different from all 2 kDa mPEG grafting concentrations, and 0, 2, 5, and 7.5 mM 20 kDa SVAmPEG ($p < 0.001$).

that mPEG grafting inhibits RSV infection in a dose-dependent manner over a broad range of viral doses. Furthermore, we also demonstrated that mPEG grafting to RSV prevented infection of polarized cells and did not dramatically disrupt the integrity of the RSV virion.

Using plaque assays we observed that the SVA-linker conferred the highest level of virus-inactivation. This is likely a result of the prolonged hydrolysis half-life of this linker chemistry. SVAmPEG has a hydrolysis half-life of 33.6 min [21], while SCmPEG and CmPEG have hydrolysis half-lives of 20.4 min [21] and ~ 10 min, respectively [22]. Thus, more activated SVAmPEG remains in solution over the 30 min derivatization reaction thereby giving rise to

enhanced grafting per viral particle. The longer hydrolysis half-life of the SVA-linker chemistry likely underlay the observed decreased effect of polymer size (2 vs 20 kDa) noted with the SVAmPEG at lower grafting concentrations.

The importance of linker chemistry is also apparent when examining previous studies with mPEG-modified adenovirus gene therapy vectors. In these studies PEGylation conditions were chosen or optimized such that modified virions remained infectious. However, similar to our findings, modification of adenovirus with high levels (or for prolonged periods) with CmPEG or succinimidyl propionate mPEG (SPAmPEG) dramatically decreased host cell infection [23,24]. Further studies indicated that the decrease in

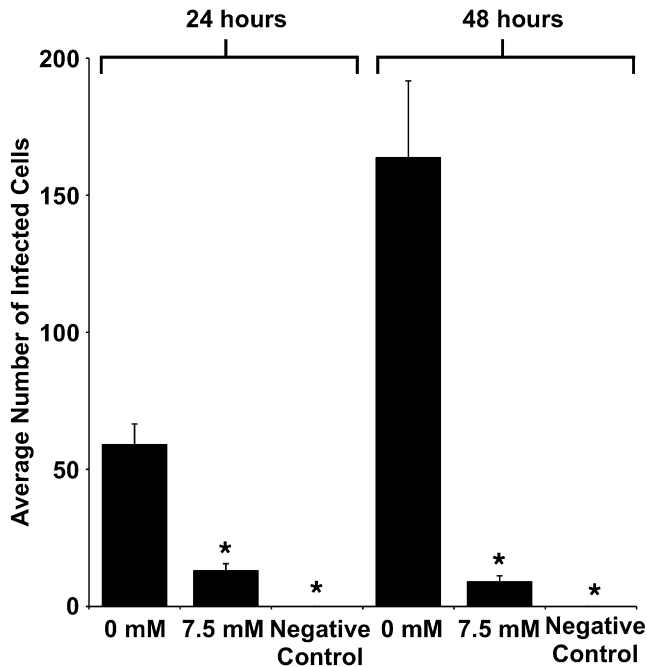


Fig. 7. mPEG-modification of polarized MDCK cells significantly inhibited rgRSV infection and proliferation. Polarized MDCK cells were modified with 7.5 mM, 20 kDa SVAmPEG and challenged with rgRSV at a MOI of 8. The high MOI was due to the decreased susceptibility of MDCK cells to RSV. Values expressed are mean \pm SE of two separate experiments performed in quadruplicate. Significance: *significantly different from the 0 mM concentration ($p < 0.001$).

infection observed with CmPEG resulted from the cross-linking of several virions by bifunctional CmPEG molecules [24]. However, in our TEM studies with SVAmPEG, little or no virus aggregates were observed. Additional studies with mPEG-modified adenovirus have also shown that modification of the virus with treysl mPEG (TmPEG) did not dramatically blunt infection both in vitro and in vivo [24–26]. These findings are not surprising as TmPEG grafted at a lower density relative to CmPEG and SCmPEG [24], and previous studies in our laboratory have shown that TmPEG is relatively poor at camouflaging cell surface epitopes on white blood cells [12]. Thus, it appears that with the TmPEG linker chemistry, high levels of protein modification are not readily achieved. As a result, the virion would not be heavily modified and would still be capable of infecting its host cell.

Polymer length, independent of linker chemistry, may also have an effect based on the structure of the virus itself. Again, as noted in Figs. 1 and 2, for both SCmPEG and SVAmPEG, shorter polymers showed a trend towards enhanced protection over the long chain polymers at lower grafting concentrations; i.e., when the virus particle to polymer ratio is low. 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 extend 11–20 nm from the virion surface [20]. Previous studies on mPEG polymers grafted to artificial surfaces have shown that 2 kDa mPEG extends \sim 6 nm from the surface [27], while 20 kDa mPEG extends \sim 50 nm from the surface (estimated length based on [27]). As mPEG is a highly flexible molecule, each grafted mPEG chain inhibits large molecules or particles (i.e. other mPEG chain, antibodies, cells) from interacting with the cell surface within the radius of the polymer. 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. This effect is further enhanced with the SVAmPEG due to its longer half-life.

The anti-viral effect of direct modification of the RSV virus with activated mPEG was not due to disruption of the virus particle itself as demonstrated by the TEM studies (Fig. 3). These findings are consistent with EM studies of mPEG-modified adenovirus, which also showed that polymer grafting did not dramatically disrupt the integrity of the virion [24]. Additionally, as demonstrated in the Fig. 1A insert, the actual ability of the modified virus to bind to target cells is significantly disrupted resulting in decreased internalization and infection.

While direct PEGylation of virus particles might be desirable and possible in some circumstances (e.g., blood banking), it is less biologically useful for respiratory viruses such as RSV. However, the ability to produce an anti-viral barrier by the direct PEGylation of the target cells (e.g., nasal epithelial cell) of the virus would be of significant clinical utility. As noted in Figs. 5–7, direct grafting of the 20 kDa mPEG polymer to mammalian cells resulted in a potent anti-RSV barrier. Indeed, at higher grafting concentrations (≥ 7.5 mM) very significant protection was observed at all MOI. However, polymer size was a crucial determinant of this anti-viral effect. In contrast to direct modification of RSV, the 2 kDa polymer demonstrated no efficacy when modifying target cells. These findings were somewhat surprising as previous studies with mPEG-modified host cells in other viruses models demonstrated some efficacy with smaller polymers although large polymers were clearly superior [13]. Furthermore, our previous studies on surface charge camouflage and antigenic masking of cell surface epitopes also showed that both short and long chain polymers conferred protection, although large polymers were similarly more effective [9,12].

The differences between our findings and those of previous studies are most likely explained by the probable interactions of RSV with its currently unknown host cell receptor. Because of the dramatic difference in host cell protection seen between the 2 and 20 kDa mPEG polymers, our findings may provide some insight as to the size of the RSV receptor. 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 [27]. In contrast, 20 kDa mPEG will project \sim 50 nm from the surface (theoretical estimate based on [27]), therefore, a much deeper mPEG zone of exclusion is created. Given the differing efficacies of the two polymer lengths, it is likely 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 20 kDa polymer, but remain unprotected by the 2 kDa polymer.

Importantly for its potential in vivo use, direct modification of polarized MDCK cells also demonstrated potent anti-RSV efficacy. Thus, it is probable that the direct mPEG-modification of polarized nasal epithelial cell will confer protection in vivo. Application of a viscous, activated mPEG gel to the nasal cavity will sufficiently modify the epithelial cells within a 3–5 minute exposure [13] to yield an effective broad spectrum anti-viral barrier. As previously noted, this anti-viral prophylaxis is effective against both enveloped and non-enveloped viruses. However, future in vivo studies will be necessary to determine if prophylactic therapy with an mPEG-based therapeutic would provide enhanced protection against RSV-mediated disease relative to current immunoglobulin based prophylactic therapies.

5. Conclusions

Our findings demonstrate that mPEG-modification of RSV or its target cells are highly effective strategies for preventing viral infection. Interestingly, while 2–20 kDa polymers demonstrated efficacy when modifying the RSV particle itself, only long chain polymers conferred protection against RSV when derivatizing the target cells. This polymer size effect was not due to a size difference between RSV (150–300 nm) and HeLa cells (11–24 μm in diameter), but potentially reflects the size of the currently unknown RSV receptor as short chain polymers were effective against other viruses [20,28]. Hence, our findings suggest that activated mPEG-based anti-viral therapies must take into consideration both the known and unknown interactions between the virus and host cell. This may be most readily accomplished using long chain polymers only, or by using a combination of long and short chain polymer species. Thus, this study further supports the potential clinical utility of mPEG grafting to host cells as a new broad spectrum, anti-viral prophylactic therapy.

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