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## Mucin biopolymers as broad-spectrum antiviral agents

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### Abstract

Mucus is a porous biopolymer matrix that coats all wet epithelia in the human body and serves as the first line of defense against many pathogenic bacteria and viruses. However, under certain conditions viruses are able to penetrate this infection barrier, which compromises the protective function of native mucus. Here, we find that isolated porcine gastric mucin polymers, key structural components of native mucus, can protect an underlying cell layer from infection by small viruses such as human papillomavirus (HPV), Merkel cell polyomavirus (MCV), or a strain of influenza A virus. Single particle analysis of virus mobility inside the mucin barrier reveals that this shielding effect is in part based on a retardation of virus diffusion inside the biopolymer matrix. Our findings suggest that purified mucins may be used as a broad-range antiviral supplement to personal hygiene products, baby formula or lubricants to support our immune system.

### Keywords

mucosal adhesion; cell culture; viral infection

### Introduction

Viral infections constitute a main class of pathogen-induced diseases. However, because of high mutation rates and the relatively high degree of dissimilarity between different types of viruses, it is difficult to develop broad-spectrum antiviral agents. Therefore, in many cases we rely on our innate immune system to repel viral infections, especially when vaccinations are unavailable or inefficient. A crucial part of the innate immune system is constituted by mucus. Mucus is a biopolymer-based hydrogel that lines all moist epithelia of humans and animals. One important function of mucus is to provide a physical barrier that prevents microbial pathogens from reaching the underlying epithelial cells<sup>1</sup>.

The exact mechanisms by which mucus prevents viral infections remain elusive. The identification of critical components of native mucus that provide antiviral activity might bear an enormous potential for the discovery of new antiviral substances. Native cervical mucus can trap various types of particles, and under certain conditions even particles that are

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smaller than the mesh size of the mucus matrix (~340 nm)<sup>2</sup>, including the herpes simplex virus-1 (HSV-1, ~180 nm) and human immunodeficiency virus-1 (HIV-1, ~120 nm)<sup>2-3</sup>. This effect is often referred to as ‘mucoadhesion’ and is based on binding interactions between the mucin biopolymers and the diffusing particles<sup>4</sup>. The major gel-forming units of mucus are mucins, large glycoproteins with molecular weights up to hundreds of kDa that carry many carbohydrate groups including *N*-acetylgalactosamine, fucose, galactose and sialic acid<sup>1b</sup>. Those carbohydrates can establish up to 70 % of the mucin mass. It appears that mucins might play a major role in the phenomenon of mucoadhesion. Indeed, isolated human salivary mucins directly interact with HIV-1<sup>5</sup> and have also been suggested to reduce HSV-1 infectivity<sup>6</sup>. Moreover, purified human breast milk mucins can block poxvirus infection<sup>7</sup>, and isolated gastrointestinal mucins inhibit rotaviruses<sup>8</sup> and noroviruses<sup>9</sup>.

Given the wide range of viruses that can be inhibited by different isolated mucins, it is possible that mucins might be able to act as broad-spectrum antiviral agents. If this is true, then mucins would be an interesting supplement for hygiene or health products, or lubricants to support the natural immune system. Obtaining large quantities of mucins from human sources such as breast milk is difficult, though. The mucosa of porcine stomachs, from which mucins can be isolated in bulk, could provide an attractive alternative source for mucin glycoproteins. Indeed, porcine gastric mucins have previously been used as a component of artificial saliva for the treatment of subjects suffering from salivary insufficiency (xerostomia)<sup>10</sup>. The antiviral properties of porcine gastric mucins, however, have not been tested yet.

Here, we explore the antiviral activity of porcine gastric mucins. We demonstrate that a solution containing purified porcine gastric mucins<sup>11</sup> can efficiently prevent infection of epithelial cells by a broad range of small mucosotropic viruses such as human papilloma virus type 16 (HPV-16), Merkel cell polyoma-virus (MCV) and a strain of influenza A virus. HPV-16 and MCV have comparable diameters of around 50 nm<sup>12</sup>, whereas a typical diameter for an influenza virion is around 100 nm<sup>13</sup>. These sizes are all significantly smaller than the mesh size of native mucus gels<sup>2</sup>. We find that reconstituted mucins do not compromise the viability of human cervical cell lines or human lung cell lines. Moreover, our results suggest that the ability of mucins to block viral infections is mainly due to a retardation of virus diffusion within the mucin solution.

## Materials and Methods

### Proteins and hydrogel reconstitution

Bovine serum albumin (BSA), matrigel (= basal lamina, a subtype of the extracellular matrix, in this article referred to as ‘ECM’) and dextran (MW: 2 MDa) were obtained from Sigma-Aldrich (St. Louis, MO), commercial mucins were purchased from Sigma Aldrich and from NBS Biologicals (Huntingdon, UK). The complex ECM Matrigel has been purified from the basal membrane of the Engelbreth-Holm-Swarm sarcoma of mice and is widely used as a model system for native ECM. A 1 % (w/v) stock solution of ECM was thawed on ice and diluted with Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) where necessary. Once applied to cells, it was incubated for 30 min at 37 °C to induce gelation. Porcine gastric mucins were purified from scrapings of fresh pig stomachs essentially as described in<sup>11a</sup>, with the exception that the cesium chloride density gradient ultracentrifugation was omitted.

To determine the molecular composition of our mucin preparation in more detail, we analyzed its content at the “Harvard Microchemistry & Proteomics Analysis Facility” by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry on a

Thermo LTQ-Orbitrap mass spectrometer. This instrument is capable of acquiring individual sequencing spectra at high sensitivity ( $\ll 1$  femtomol) for multiple peptides in the chromatographic run. The spectra are then correlated with known sequences using the algorithm Sequest<sup>14</sup>.

The analysis showed that our mucin preparation contains the following mucins: MUC5AC, MUC2, MUC5B and MUC6. As expected for the stomach mucosa, MUC5AC seems to be the major component of our purification. Other proteins within our mucin preparation include histones, actin, and albumin. Yet, as this mass spec technique is not suitable to determine exact protein concentrations, we can currently not establish the relative concentrations of all those proteins.

For reconstitution, lyophilized mucins were hydrated overnight at 4 °C in distilled water. The homogeneous mucin solutions were buffered to the desired pH with acetate buffer calibrated to pH 3 or HEPES buffer calibrated to pH 7. The final buffer concentration in the mucin solution was 20 mM and the ionic strength was adjusted to 20 mM with NaCl. BSA, dextran and commercial mucins were treated the same way as our purified mucins and hydrated in either pH 7 HEPES buffer containing 20 mM NaCl, or in PBS.

### Virus particles

HPV-16 and MCV reporter vectors (pseudoviruses) carrying GFP reporter plasmids and fluorescently labeled virus-like particles (VLPs) were generated according to previously-reported methods<sup>15</sup>. In brief, 293T cells were co-transfected with expression constructs encoding the capsid genes with or without a reporter plasmid encoding GFP. Plasmids used in this work are available through Addgene.org and detailed protocols are posted at <http://home.ccr.cancer.gov/LCO/>. The influenza virus used here is the A/WSN/1933 (H1N1) strain, which is a commonly used influenza lab strain. For this study, the virus has been modified such that the PB1 segment carries the green fluorescent protein (GFP) in place of the PB1 coding sequence, as described in<sup>16</sup>. As a consequence, these influenza viruses are only able to replicate in engineered cell lines that express the PB1 protein.

HPV-16 VLPs were conjugated to Alexa Fluor 488 according to Alexa Fluor 488 Protein Labeling Kit (Invitrogen #A10235) instructions. Clarified cell lysate containing VLPs was diluted with water to a total protein concentration of 3 mg/ml, adjusted to 0.1 M sodium bicarbonate and added to a vial of Alexa Fluor 488 reactive dye provided with the kit. The conjugated VLPs were adjusted to neutral pH with sodium phosphate and then purified over an Optiprep gradient. Infectivity monitoring using an encapsidated *Gaussia* Luciferase plasmid indicated no detectable alteration of the particle-to-infectivity-ratio of Alexa Fluor 488 conjugated VLPs, indicating that the dye conjugation reaction did not have a major impact on capsid protein function<sup>17</sup>.

### Cell culture

Human cervical cells (HeLa) were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with L-glutamine, 10% fetal bovine serum (FBS), 4.5 g/L glucose, and 25 U/mL penicillin, 25 µg/mL streptomycin at 37 °C in a humidified atmosphere at 5 % CO<sub>2</sub>. The human alveolar epithelial cell line A549 was cultured in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with L-glutamine, 5 % FBS, 4.5 g/L glucose, and 25 U/mL penicillin, 25 µg/mL streptomycin at 37 °C in a humidified atmosphere at 5 % CO<sub>2</sub>. As an infection target for the influenza virus, a variant of Madin-Darby canine kidney cells, MDCK-SIAT1-CMV-PB1, was used. These highly adherent epithelial cells have been modified by lentiviral transduction to constitutively express the PB1 protein under a CMV promoter to allow the modified influenza viruses used in this study to efficiently replicate

inside these cells<sup>16</sup>. MDCK cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with L-glutamine, 10% fetal bovine serum (FBS), 4.5 g/L glucose, and 25 U/mL penicillin, 25 µg/mL streptomycin at 37 °C in a humidified atmosphere at 5 % CO<sub>2</sub>. Cell counts were performed using a hemocytometer.

### In vitro infection assay

For *in vitro* infection,  $5 \times 10^4$  HeLa cells,  $7.5 \times 10^3$  A549 cells, or  $3 \times 10^4$  MDCK-SIAT1-CMV-PB1 cells were seeded into each well of a 96-well microtiter plate (BD Biosciences, San Jose, CA) and allowed to adhere overnight. For MDCK-SIAT1-CMV-PB1 cells, three hours prior to infection the DMEM was replaced by OptiMem media (Invitrogen, Carlsbad, CA) supplemented with 0.01 % FBS, 0.3 % BSA, 100 U/mL Penicillin, 100 µg/mL Streptomycin, and 100 µg/mL CaCl<sub>2</sub>. For infection, the cell culture medium was removed, and replaced with 50 µL of a biopolymer solution (ECM, mucin, BSA, or dextran). As controls, cells were lined with DMEM, PBS or HEPES buffer (20 mM HEPES, 20 mM NaCl, pH 7). For infection, 5 µL of virus solution was carefully added to each well (Fig. 1) and incubated for two hours at 37 °C. This mimics the approximate time span after which a mucus layer is renewed *in vivo*, owing to the synthesis of fresh mucins on the epithelial surface and subsequent shedding of the “old” mucus layer together with trapped particles. In our *in vitro* setup, the virus particles are allowed to translocate through the biopolymer solution. This process will be driven by a combination of diffusion, turbulences created by adding a drop of virus solution onto the biopolymer layer, and thermal convection effects. After this incubation step, the cells were rinsed three times with 150 µL PBS. This washing step removes the biopolymer solution together with any viruses that have not yet attached to the cell monolayer. Then, 150 µL cell culture medium was applied and the cells were cultured for 16 hrs (for MDCK-SIAT1-CMV-PB1) or for two more days (for HeLa and A549), respectively, to allow for expression of GFP. For FACS analysis, the adherent cells were harvested by trypsinization and analyzed using a LSRII flow cytometer (BD Biosciences, San Jose, CA) or an Accuri C6 flow cytometer (Accuri Cytometers, Cambs, UK). Uninfected control cells were used to define the base-line autofluorescence of each cell batch. Thresholds in the analysis software (FACSDiva version 6.1.2, BD Biosciences, San Jose, CA, or CFlow Plus, Accuri Cytometers, Cambs, UK) were chosen in such a way that 0.1 % of the control cells were counted as GFP-positive. Relatively high viral doses were used to minimize assay variability we sometimes observed with multiplicities of infection below 1. All experiments were performed in triplicates.

### Cell toxicity assay

For assessing putative cytotoxic effects of mucins, cells were incubated with a 1 % (w/v) pH 7 mucin solution for 2 hrs, followed by washing with PBS. The cells were then incubated with culture medium for 48 hrs. Then, the percentage of viable cells was determined using a live/dead kit for mammalian cells (Invitrogen, Carlsbad, CA). In brief, cells were stained with two dyes, calcein and Ethidium homodimer-1, which emit green and red fluorescence, respectively. Cells that emit green fluorescence only are considered viable, whereas red fluorescence is a marker for cytotoxic effects. 2 µM calcein and 2 µM Ethidium homodimer-1 were suspended in the appropriate cell media and incubated with the cells for 20 min. Images were acquired on an Axio Observer microscope (Zeiss, Oberkochen, Germany) with an EC-Plan Neofluar 10× 0.3 NA lens (Zeiss). Cell counts were performed with the image analysis software ImageJ using a cell count plug-in.

### Single particle tracking

For single particle tracking experiments, mucins (or dextran, or BSA) were hydrated as described before. Fluorescently labeled HPV-16 particles were added to each biopolymer solution, and ~30 µL of the virus/biopolymer solution was placed into an open 10 mm × 10

mm square of vacuum grease which has been deposited onto a glass slide. This custom made sample chamber was then sealed with a cover slip. Brownian motion of virus particles was followed at room temperature on an Axio Observer microscope (Zeiss, Oberkochen, Germany) with an EC Plan 40× 0.75 NA PH2 objective (Zeiss) using fluorescence microscopy. Movies were acquired with a digital camera (ORCA-R2, C10600; Hamamatsu, Hamamatsu City, Japan) at 10 frames/s and processed with the software OpenBox<sup>18</sup>. For diffusing virus particles it is highly difficult to obtain coherent trajectories  $\mathbf{r}(t) = (x(t), y(t))$  due to the weak contrast within the biopolymer solutions and the high virus mobility in certain samples. Thus, the calculation of a full MSD curve,  $\text{MSD}(\tau) = \sum [\mathbf{r}(i\Delta t + \tau) - \mathbf{r}(i\Delta t)]^2$ , as typically applied to single particle tracking data is not feasible here. Instead, from our movies we measured the squared distance  $\delta^2 = [\mathbf{r}(t_0+1s) - \mathbf{r}(t_0)]^2$  a given virus particle travels by diffusion within 1 s of observation time – corresponding to 10 frames, i.e. a time span that most of the virus particles can be followed coherently. We then calculated the average  $\langle \delta^2 \rangle$  by pooling measurements from each sample, i.e. data for up to 50 different virus particles and different starting time points  $t_0$ . This yields a quantity that represents the mean square displacement of the particle ensemble for a lag time of  $\tau = 1s$ ,  $\langle \delta^2 \rangle = \text{MSD}(1s)$ . It should be noted that, due to the limited statistics, this quantity cannot be used to determine an accurate diffusion coefficient. It gives, however, a reasonable measure for comparing the diffusion behavior of the VLPs in different microenvironments.

### Viscosity measurements

The dynamic viscosity of the biopolymer solutions was measured with a stress controlled rheometer (AR-G2, TA instruments, New Castle, USA) with a 40 mm cone-plate geometry (2° cone angle) and 56  $\mu\text{m}$  truncation distance. Approximately 500  $\mu\text{L}$  of a biopolymer solution was loaded onto the rheometer, subjected to a shear rate ramp in the range of 100/s – 1000/s and the viscosities measured in this shear rate range were averaged.

## Results

### Hydrogels can protect cells from viral infection by trapping the viruses in the biopolymer matrix

For initial experiments we used reporter pseudovirions based on HPV-16, an HPV type that causes a majority of cases of cancer of the uterine cervix<sup>19</sup>. As targets for HPV-16 infection we chose HeLa cells, which originate from human cervical tumor tissue. *In vivo*, prior to infection of the target cells, HPV-16 attaches to the basal lamina, a complex multi-component hydrogel that supports the basal cells of the cervical epithelium<sup>20</sup>. This attachment process requires binding interactions between the HPV-16 capsid proteins and the glycosaminoglycan heparan sulfate, which is one of the main components of the basal lamina<sup>21</sup>.

Indeed, we were able reproduce this adsorption of the virus *in vitro* by using a basal lamina hydrogel (here referred to as extracellular matrix (ECM), see Materials). This hydrogel is a complex mixture of the biopolymers collagen IV, laminin and heparan sulfate. In the concentration regime used here, the ECM hydrogel has a mesh size on the order of a few microns<sup>22</sup>. Previous experiments have shown that particles much smaller than this mesh size can be efficiently immobilized in the ECM biopolymer matrix. This immobilization effect is, in part, established by the heparan sulfate glycosaminoglycan and due to electrostatic binding interactions between the diffusing particles and the hydrogel biopolymers<sup>22</sup>.

Here, we find that an 0.25 % ECM hydrogel also suppresses the diffusive motion of HPV-16 pseudovirions (Fig. 2A), and we measured a very low diffusion coefficient of  $D_{\text{HPV,ECM}} =$



$(8 \pm 2) \times 10^{-4} \mu\text{m}^2/\text{s}$ . This value is  $\sim 10,000$  times smaller than the corresponding value for pseudovirion diffusion in buffer control conditions which we calculated to be  $D_{\text{HPV,water}} = 9.6 \mu\text{m}^2/\text{s}$  using the Stokes-Einstein relation,  $D(R) = k_B T / (6 \pi \eta R)$ . There,  $k_B T$  denotes the thermal energy,  $\eta$  the viscosity of water and  $R$  the radius of the HPV-16 virus particle.

We then hypothesized that a hydrogel, which can trap virus particles inside the biopolymer matrix, should also be able to protect underlying cells from viral infection. To test this hypothesis, we lined a HeLa monolayer with either DMEM or with an ECM hydrogel and then exposed the culture to HPV-16 pseudoviruses (Fig. 1 and methods). Indeed, a 0.25 % (w/v) ECM gel reduced the percentage of infected HeLa cells about 6-fold compared to the positive control (Fig. 2B). The shielding efficiency of ECM becomes stronger at higher biopolymer concentrations where the HPV-16 infection rate is reduced to only a few percent. Together, these data demonstrate that ECM hydrogels have the capacity to effectively prevent viruses from infecting underlying cells, and that this is achieved by trapping the viruses in the biopolymer matrix.

### Reconstituted mucin solutions do not compromise the viability of HeLa cells

In the remainder of this article we aim at testing whether purified porcine gastric mucins can also serve as an anti-viral infection barrier. However, before assessing the impact of porcine gastric mucins on the diffusion behavior and infectivity of HPV-16, we verified that reconstituted mucin solutions are non-toxic for HeLa cells (see Methods). We found that HeLa cells exposed to the mucin solution showed a viability that was comparable to cultures exposed to the HEPES buffer control (Table 1). Similar results were obtained for commercial porcine gastric mucins purchased from Sigma Aldrich. However, commercial mucins obtained from another vendor (NBC Biologicals, Huntingdon, UK) appear to induce strong cytotoxic effects as the corresponding percentage of viable cells was very low. We conclude that our manually purified mucin solutions constitute a biocompatible material that does not compromise the viability of HeLa cells.

### Mucin solutions retard HPV-16 diffusion

If porcine gastric mucins are supposed to act as an infection barrier towards HPV-16 viruses, then trapping of virus particles inside the mucin matrix would be a possible mechanism to achieve this goal, similar to what we observed for ECM hydrogels. Thus, we next analyzed the diffusion behavior of individual fluorescently labeled HPV-16 VLPs in mucin solutions using single particle tracking microscopy (Fig. 3A and Methods). In HEPES buffer, HPV-16 diffused rapidly and it was difficult to follow a given VLP for an extended time period as most of the virus particles left the focal plane during the observation time. In contrast, in a 1 % mucin solution, the diffusive motion of HPV-16 VLPs was significantly suppressed. As depicted in Fig. 3A, this retardation of HPV-16 diffusion is even more pronounced when the pH level of the mucin solution is lowered from pH 7 to pH 3. At the acidic pH, where the mucin biopolymers form a hydrogel<sup>11b</sup>, the VLPs appeared to be completely immobilized. Importantly, this lockdown of virion mobility appeared to be a global effect since we did not detect any mobile VLPs in the pH 3 sample.

To better quantify the observed differences in the VLP diffusion behavior, we determined the mean square displacement for an ensemble of up to 50 virus particles per condition at a lag time of 1 s (see Methods). As depicted in Fig. 3B, this ensemble quantity reflected the pronounced differences observed in the trajectories of single virus particles. In other words, mucin solutions were able to efficiently trap the VLPs both at neutral and acidic pH. This suggests that mucin solutions should also be able to employ this trapping of HPV-16 virus particles to act as an infection shield in our *in vitro* infection assay.

### Mucin solutions pose a barrier towards HPV-16 infection

In the next step, we evaluated the performance of porcine gastric mucins as a barrier towards viral infection. When HEPES buffer is used as the sole protective layer, more than 65 % of the cells were infected (Fig. 4A). In contrast, when the cells were covered with a 1 % (w/v) mucin solution prior to virus inoculation, the proportion of infected cells was decreased to ~6 %. The shielding efficiency of the mucin solution depended on the mucin concentration used (Fig. 4A), confirming previous findings that the permeability of mucin solutions can be regulated by the mucin concentration<sup>23</sup>, in analogy to native mucus<sup>24</sup>. This demonstrates that purified mucins can indeed pose an efficient barrier towards HPV-16 infection. Interestingly, commercial porcine gastric mucins obtained from Sigma Aldrich are less efficient in reducing HPV-16 infection when used at a similar concentration as our manually purified mucins (Table 1). Similarly, also particle diffusion experiments have shown that commercially purified mucins do not establish the same diffusion control as native intestinal mucus<sup>25</sup>. Our findings suggest that the detailed mucin purification protocol and mucin biochemistry might be important for the performance of mucins as a barrier towards viral infection.

### Mucin solutions form more efficient diffusion and infection barriers than BSA or dextran solutions

Can any solution of macromolecules protect cells from viral infections or are certain biopolymer properties required to achieve this effect? To tackle this question, we compared our results obtained for 1 % (w/v) mucin solutions to two other biopolymer solutions: a bovine serum albumin (BSA, MW ~ 66 kDa) solution and a dextran solution (MW ~ 2 MDa), which were both prepared at the same concentration as the mucin solution, i.e. at 1 % (w/v) (Fig. 5A). When used as a shielding layer in our *in vitro* infection assay, BSA did not cause a major reduction in the infectivity of HPV-16, and dextran was only moderately inhibitory (Fig. 5B). Moreover, neither the BSA solution nor the dextran solution appeared to significantly inhibit HPV-16 diffusion (Fig. 3A and B). This suggests that the inhibitory effect of dextran observed in Fig. 5B is not simply due to a reduction of virus diffusion, but rather that dextran may modestly inhibit a subsequent step in the infection process. These results suggest that unique chemical/physical properties of the mucin biopolymer may be needed for efficient virus trapping and infection blocking.

### Mucin solutions also pose a barrier towards influenza and MCV infection

We next investigated whether the shielding effect of mucin solutions is specific for HPV-16 or whether mucins can prevent other small viruses from infecting epithelial cells as well. To address this question we repeated the *in vitro* infection experiments described before, but this time using influenza viruses (100 nm). Influenza A belongs to the virus family *Orthomyxoviridae* and infects the epithelia in the respiratory tract of humans. The introduction of new influenza subtypes into the human population can cause pandemics, including the deadly 1918 pandemic (which is estimated to have killed over 40 million people worldwide<sup>26</sup>) and more recently the 2009 swine-origin H1N1 pandemic<sup>27</sup>. As targets for infection with the A/WSN/33 (H1N1) strain of influenza used in this study, we chose modified canine kidney cells (MDCK-SIAT1- PB1, see Methods). The viability of this cells type was also unaffected by our 1 % (w/v) mucin solution (Table 1).

With our *in vitro* infection assay, we found a similar ability of mucins to block influenza infection as observed for HPV-16 pseudoviruses (Fig. 4B). A comparable dependency of this infection barrier efficiency on the mucin concentration was obtained when HEPES or PBS was used as a buffer for mucin reconstitution, respectively (Fig. 4B). Furthermore, the inhibitory profiles of BSA, dextran and mucin towards influenza infection resembled those

obtained for the HPV/HeLa cell pair, with mucins being much more efficient than BSA or dextran (Fig. 5C).

As a third virus species, we also investigated the Merkel cell polyomavirus (MCV, ~50 nm), which is believed to infect both the skin and the oral mucosa<sup>28</sup>. MCV is a member of the viral family *Polyomaviridae* and is thought to trigger Merkel cell carcinoma, an aggressive form of skin cancer. MCV pseudoviruses can successfully transduce A549 cells, a cell line established from a human lung epithelial tumor. With this virus/cell pair, we observed the same trend in the protective ability of BSA, dextran, and mucin solutions as for the HPV-16/HeLa and the influenza/MDCK combinations (Fig. 5D). In agreement with our previous findings on HeLa and MDCK cells, we also observed that the viability of A549 lung cells was not compromised by exposure to a 1 % (w/v) mucin solution (Table 1) underscoring the biocompatibility of our purified mucin biopolymers.

Together, these data demonstrate that the mucin biopolymer matrix imposes an efficient infection barrier not only towards HPV-16 but also towards influenza and MCV.

### High NaCl concentrations increase the barrier function of mucins

Rinsing the nose or the oral cavity with saline solutions is a traditional household remedy for the common cold. Thus, in a final step, we asked how different concentrations of sodium chloride in the mucin hydration buffer would affect the inhibitory activities of mucin solutions towards viral infection. For this set of experiments we chose the influenza/MDCK cell pair, as only MDCK cells showed strong enough adhesion to allow us performing our *in vitro* infection assay in the presence of high NaCl concentrations.

When mucins were hydrated in low salt HEPES buffer (20 mM NaCl), the concentration dependent inhibitory activity of mucins was comparable to the situation when PBS (140 mM NaCl and minor amounts of divalent ions) was used for mucin hydration (Fig. 4B). Accordingly, also HEPES buffer containing 150 mM NaCl returned comparable results when used as a hydration medium for 0.25 % (w/v) and 1 % (w/v) mucin solutions (Fig. 6A and B). However, when the NaCl concentration of the HEPES buffer was increased to 300 mM (Fig. 6A), we detected a significant enhancement of the influenza inhibition efficiency provided by a 1 % (w/v) mucin solution. This effect is not due to an altered base-line infectivity or reduced virus stability, as the control samples were unaffected by the change in the ionic strength of the mucin hydration buffer. A similar trend, albeit much stronger, was observed when the NaCl concentration was varied in a 0.25 % (w/v) mucin solution. At this low mucin concentration, the inhibitory activity of the mucin solution was drastically enhanced by raising the NaCl concentration (Fig. 6B). Surprisingly, at 500 mM NaCl, the low concentration mucin solution was as efficient in reducing influenza infectivity as the 1 % (w/v) mucin solutions discussed before.

These results demonstrate that the ability of mucin solutions to act as an anti-viral infection shield can be strengthened by high NaCl concentrations.

## Discussion

Our data reveal that purified porcine gastric mucins can efficiently shield an underlying cell monolayer from infection by viruses as small as 50 nm in diameter. This effect can be achieved with mucin concentrations as low as 0.25 – 1 % (w/v) which is 4–5fold lower than the mucin concentrations found in native mucus<sup>29</sup>. Data obtained at the single particle level suggest that the barrier function of mucin solutions is achieved in part by suppressing the mobility of virus particles and trapping them inside the biopolymer matrix. Previous studies on virus diffusion in native mucus have suggested that this immobilization effect is based on



adhesive interactions with certain mucus components, and a similar mechanism – in addition to geometric hindrance effects – might also be responsible for the virus trapping described here. However, owing to the lower mucin concentration, the mesh size of our reconstituted mucin system should be larger than the literature value of 300 nm reported for native cervical mucus and thus significantly larger than the diameter of the viruses studied here. This suggests that geometric hindrance is not likely to be the main mechanism by which mucin solutions trap virus particles.

The anti-viral activity of mucins is still observable when the mucin layer is removed from the cells by washing prior to virus inoculation (data not shown). It is possible that mucin polymers remain on the cellular surface after the washing step, and that these could act as a passivation agent by blocking the cell receptors required for virus binding thus preventing successful cell infection. Such a cell membrane passivation mechanism might also explain the modest inhibitory effect of dextran and even BSA mentioned earlier and could constitute a second anti-viral defensive mechanism provided by mucins besides virus trapping. Alternatively, this could indicate that, indeed, the interaction with mucins affects cell physiology, perhaps by modulating the cytoskeleton, or by other, entirely unrelated cellular mechanisms that render cells more resistant toward viral infection.

We have shown that the trapping of HPV-16 inside the mucin matrix is more efficient at low pH. This agrees with previous findings on particle diffusion and particle translocation experiments in reconstituted mucin solutions<sup>23, 30</sup> and underlines the possibility to tune the permeability of this biopolymer based diffusion barrier by pH. We emphasize that our single particle tracking assay might still underestimate the virus trapping effect established by mucins: the residual mobility observed for HPV viruses in mucin solutions could represent thermal undulations of the mucin biopolymers to which the virus particles are bound, rather than a true local diffusion of the virus particles.

Which components of reconstituted mucin solutions could be responsible for trapping virus particles inside the biopolymer matrix? One commonality of the viruses studied here is their interaction with sugar groups during the infection process. HPV-16, for example, targets negatively charged heparan sulfate chains in the ECM or on the cell surface. MCV appears to use heparan sulfate and sialylated glycans as receptors<sup>17, 31</sup>. Influenza attaches to cells via binding of the hemagglutinin protein to sialic acid moieties on the host cell. Mucins contain polyanionic sugars including sialic acid<sup>1b</sup>, which may serve as decoys for the various receptors utilized by the three virus families we studied here. It is interesting to note that commensal and pathogenic microbes appear to specifically target such sugar moieties when they are trying to weaken the native mucus barrier by enzymatic degradation<sup>32</sup>, and that the influenza neuraminidase protein can cleave sialic acid moieties<sup>33</sup>. This supports the notion that those sugar moieties indeed play an important role in the defense mechanism of mucins towards pathogens.

Whereas the detailed mechanistic and molecular principles that establish the adhesive effects between viruses and mucins are still to be deciphered, the adsorption of the virus particles to mucins could, at least in part, be due to relatively non-specific electrostatic interactions with either the sugar moieties or with the protein backbone of the mucin biopolymers (Fig. 7). Electrostatic interactions have already been suggested to contribute to the trapping of polystyrene particles inside reconstituted mucin solutions<sup>23</sup>. However, other physical interactions such as van-der-Waals forces or hydrogen bonds could also cause trapping of viruses inside the mucin matrix. Indeed, our finding that high NaCl concentrations strengthen the barrier function of mucin solutions demonstrates that electrostatic binding interactions are not sufficient to explain the barrier properties of mucins towards virus particles. At high ionic strength, the electrostatic interactions between the virus capsids and

the mucin biopolymers should be sufficiently weakened by Debye screening. Thus, if binding of influenza viruses by mucins were based on electrostatic interactions only, then high ion concentrations would be expected to reduce the barrier function of mucins towards influenza rather than strengthen it.

In native mucus such as saliva, certain ions such as  $\text{Ca}^{2+}$  increase the macroscopic viscosity of the hydrogel and retard the diffusion of tracer particles<sup>34</sup>. The viscosity of our mucin solutions, however, is reduced by  $(12 \pm 7)$  % at 500 mM NaCl compared to low salt conditions. Therefore, an altered viscosity of our mucin solution cannot account for the enhanced influenza inhibition of mucin solutions at high NaCl concentrations. Instead, we speculate that multiple low-affinity bonds (based on e.g. polar and/or hydrophobic interactions) between the mucin sugar groups and the virus capsids might be responsible for the trapping of the virus particles. Which combination of physical forces regulates these binding interactions and how they depend on the detailed buffer milieu is a complex question that will need to be addressed in detail in future experiments.

Once the detailed biochemical motifs in the mucin biopolymer are determined that are responsible for binding virus particles, then mucin biopolymers might also serve as a good role model for the *de novo* synthesis of engineered biopolymer materials, which trap virus particles and thus act as an artificial shielding layer towards viral infection. Indeed, a recent study employs mucin-like polysaccharide motifs in engineered polymers to trap HIV viruses in the polymer matrix<sup>35</sup>. The *in vitro* infection assay presented here provides a suitable platform for the screening of such engineered hydrogels to evaluate both their biocompatibility and their efficiency as an infection barrier in one convenient format.

So far, viral translocation through mucus has been studied essentially by two methods, namely single particle tracking<sup>3</sup> and fluorescence recovery after photobleaching<sup>36</sup>. Here, we present a third approach, an *in vitro* infection assay that measures bulk translocation of virus particles through mucin solutions, or other biopolymer materials. This assay has several advantages compared to single particle tracking studies. First, it does not require fluorescence labeling of the virus capsid. Such a labelling is mandatory for optical tracking experiments with such small particles but might introduce artifacts if the label enhances or reduces adhesive interactions with the biopolymers. Second, the assay measures the bulk permeability of a biopolymer solution or a hydrogel by analyzing the infection status of a whole underlying cell layer. Thus, the infection assay directly takes into account putative heterogeneities in the hydrogel architecture, which otherwise can require numerous time-consuming single particle measurements for their detection.

## Conclusion

Due to their antiviral activity, biocompatibility and availability in relatively large quantities, isolated porcine gastric mucins might be suitable candidates for supplements in personal hygiene products such as mouth rinse or tooth paste. They might also be good additives for wound treatment ointments or for genital lubricants, where they could help protecting our inner or outer body surfaces from viral infection. Porcine gastric mucins can be purified in bulk, and they are already used as components for artificial saliva. Their ability to block human pathogenic viruses might not be limited to HPV-16, MCV, and influenza but could potentially also apply to other viruses such as HIV, HSV, and Hepatitis B or C. Thus we envision porcine gastric mucins to be promising antiviral components for future biomedical applications.

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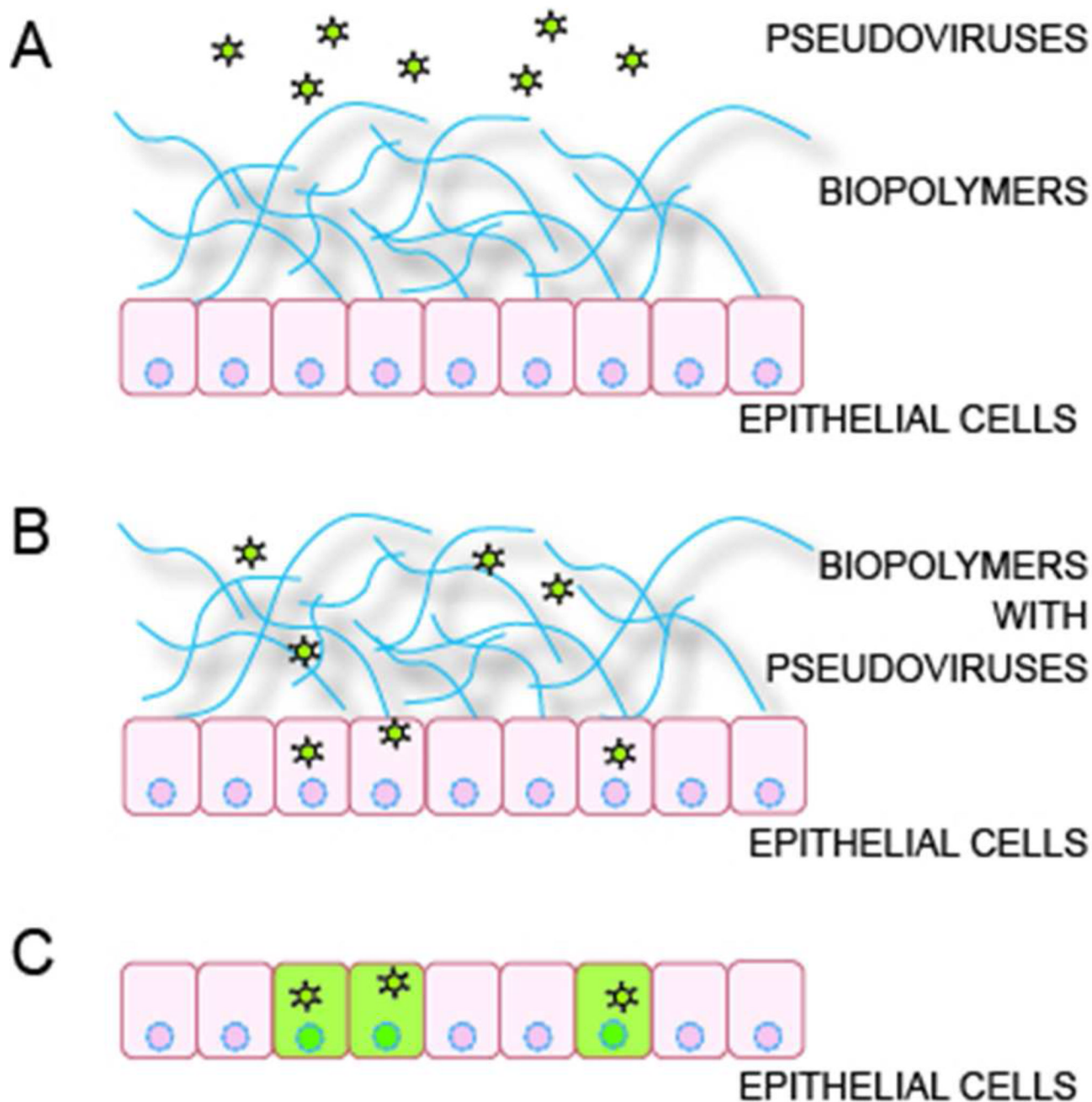
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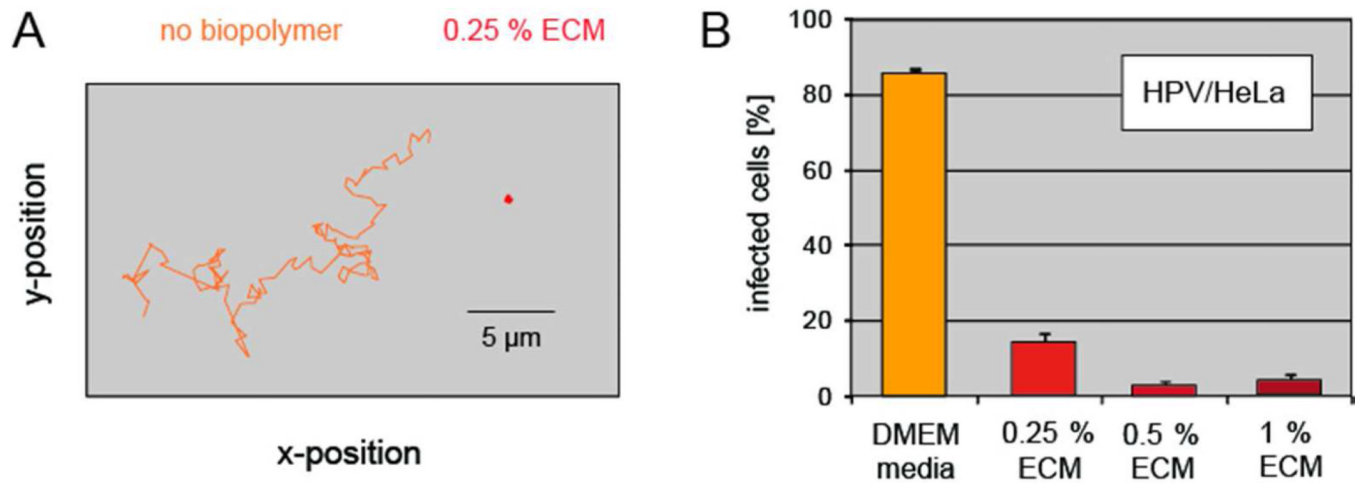
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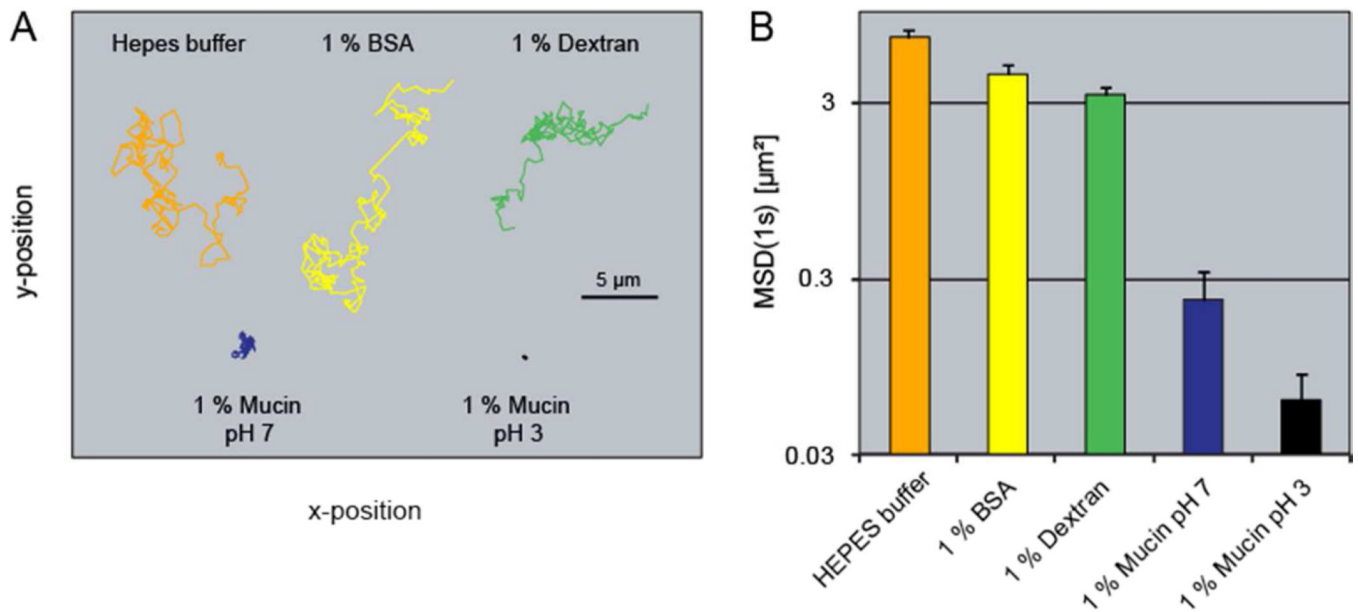


**Figure 1.** Schematic representation of the *in vitro* infection assay used in this study. (A) A monolayer of suitable target cells is lined with a biopolymer solution which is then exposed to a small drop of a virus solution. (B) The cells are incubated with the biopolymer solution and the viruses for 2 h. During this time the viruses may spread through the biopolymer solution and infect the underlying cells. (C) The biopolymers and remaining viruses are removed by washing with PBS and the cells are incubated for 48 h. All viruses used in this study carry a gene encoding GFP, allowing for distinguishing infected cells (GFP-positive) from uninfected cells (GFP-negative) by flow cytometry.



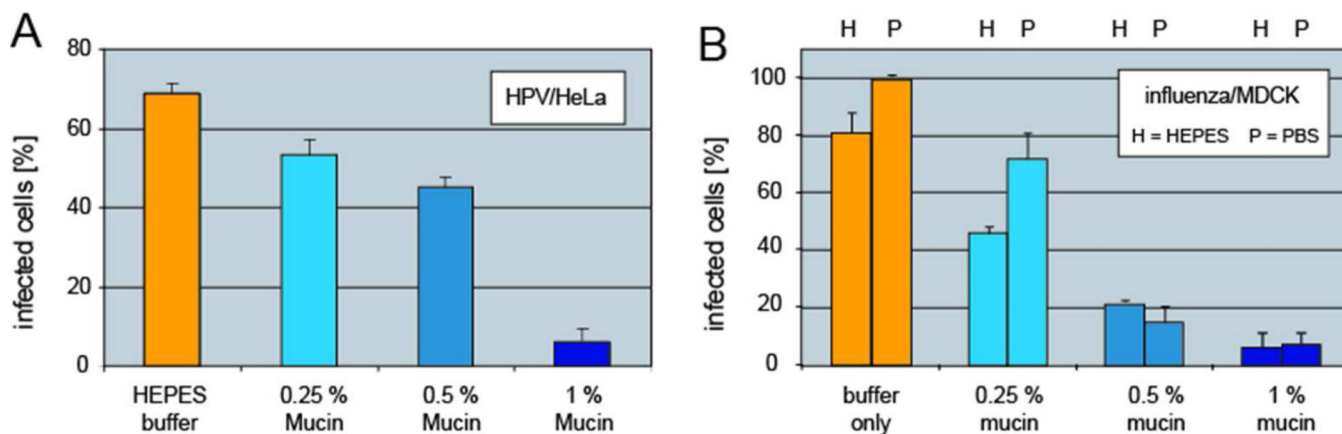
**Figure 2.**

Basal lamina (ECM) hydrogels trap HPV-16 viruses and act as an infection barrier. (A) Trajectories of ~20 s duration for HPV-16 VLPs in the absence of biopolymers (orange) and in 0.25 % (w/v) ECM (red). (B) Percentage of HPV-16-infected HeLa cells using ECM hydrogels at increasing biopolymer concentrations as an infection shield. Error bars denote the error of the mean from three independent experiments.

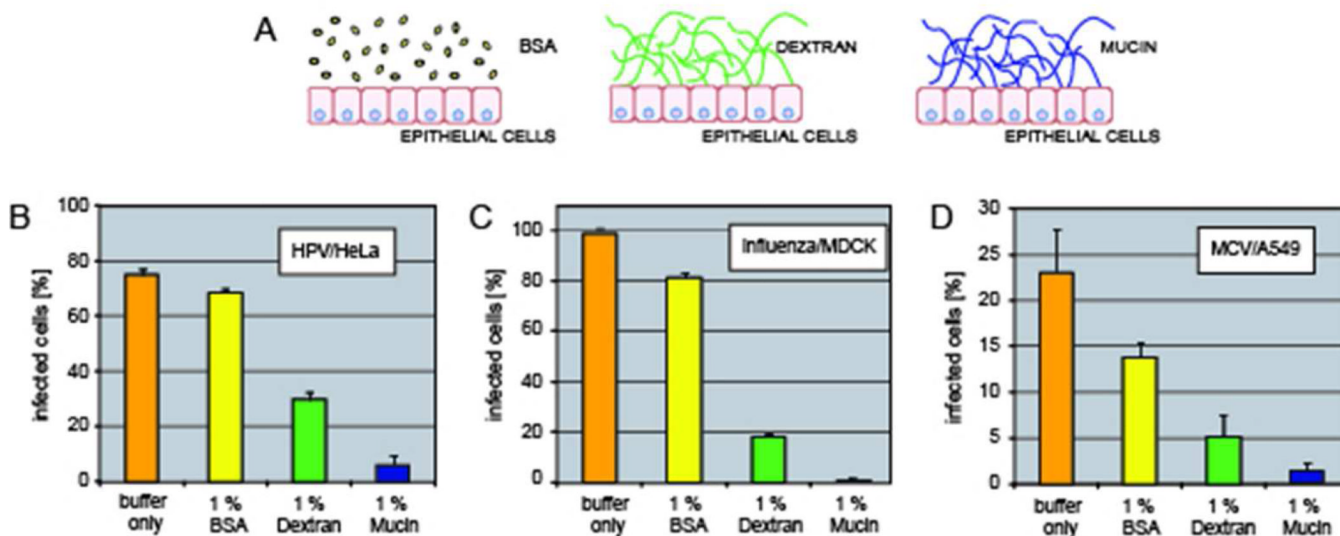


**Figure 3.**

The diffusive motion of HPV-16 VLPs depends on the microenvironment. (A) Trajectories of 20 s duration for single HPV-16 VLPs in different biopolymer solutions. (B) Averaged square displacement of HPV-16 VLPs calculated for a diffusion time of 1 s (see Methods). HPV-16 diffusion is significantly reduced in 1 % (w/v) mucin at pH 7 and massively suppressed in 1 % (w/v) mucin at pH 3. The error bars denote the error of the mean. The number of viral particles analyzed was N=13 for HEPES buffer, N=22 for BSA, N=26 for dextran, N=54 for mucin pH 7 and N=51 for mucin pH 3.

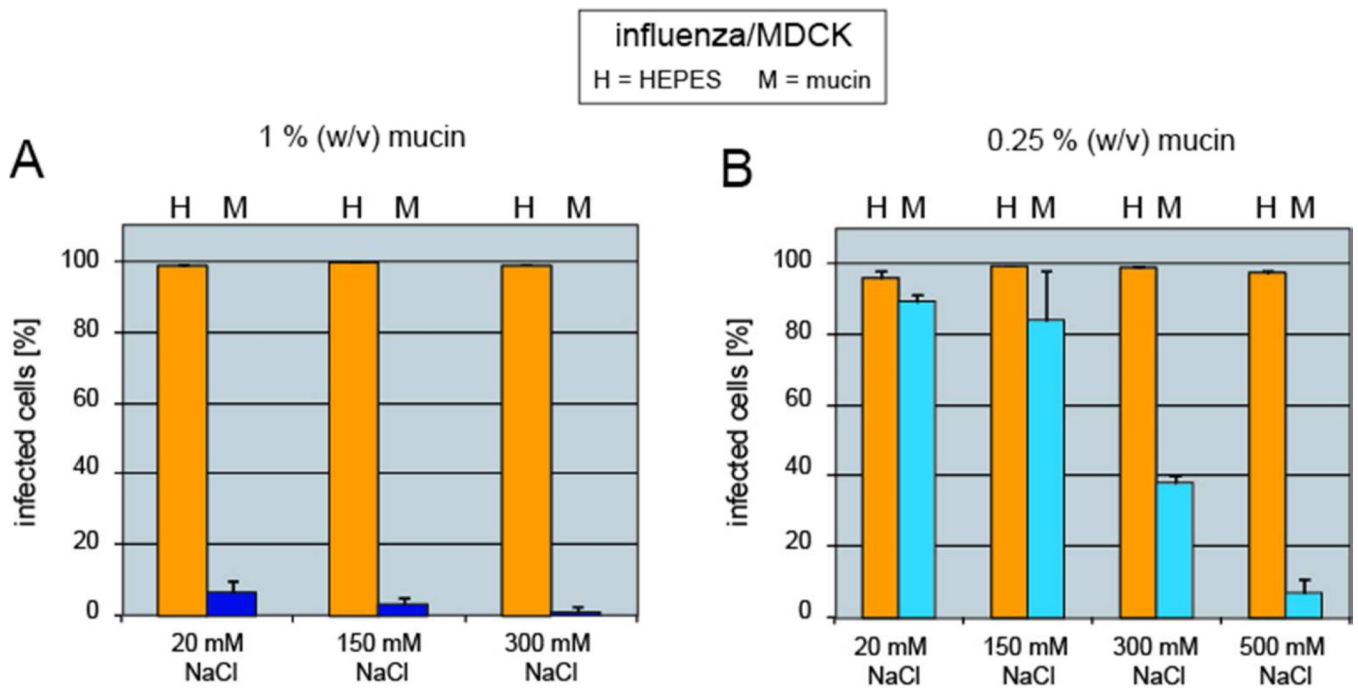


**Figure 4.** The efficiency of mucin solutions as a shielding layer towards viral infection depends on the mucin concentration. Suitable cells are employed as an infection target, and the percentage of infected (= GFP-positive) cells is determined by flow cytometry. With increasing mucin concentration, the percentage of HeLa cells infected by HPV-16 (A) and the percentage of MDCK cells infected by influenza (B) is decreased from ~70–80 % to ~6 %. A similar trend is obtained if PBS is used as a mucin hydration buffer instead of HEPES (B). Error bars denote the error of the mean from three independent experiments.



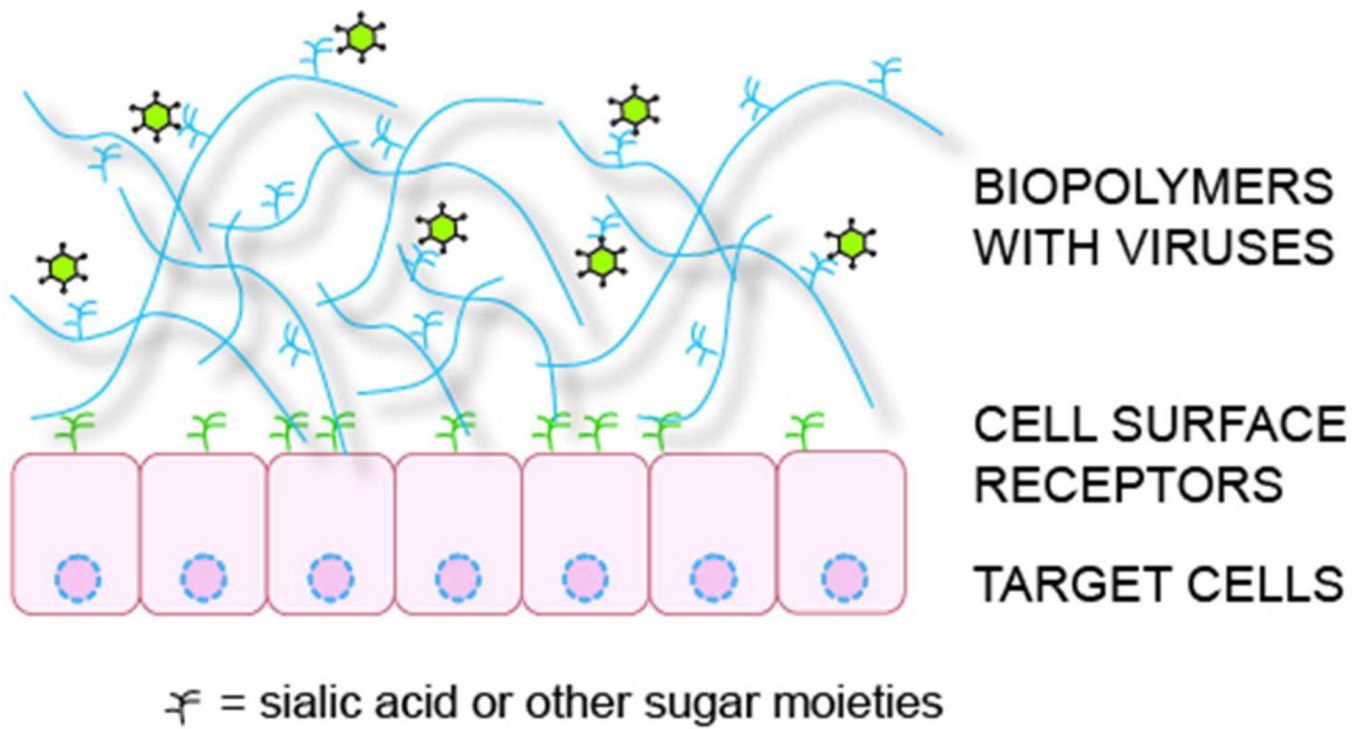
**Figure 5.** Mucin solutions form more efficient infection shields than BSA or dextran solutions. (A) Pictograms of epithelial cells that are lined with a BSA, dextran or mucin solution. The percentages of infected cells in the presence of these different biopolymers are depicted for the HPV-16/HeLa pair in (B), for the influenza/MDCK pair in (C), and for the MCV/A549 pair in (D). As a reference, buffer w/o biopolymers is used. Error bars denote the error of the mean as obtained from three independent experiments.





**Figure 6.**

The efficiency of mucin solutions to block influenza infection depends on the NaCl concentration of the mucin reconstitution buffer. The percentage of MDCK cells infected by influenza decreases with increasing NaCl concentrations both for 1 % (w/v) mucin solutions (A) and for 0.25 % (w/v) mucin solutions (B). In the latter case, the enhancement in the protective ability of the mucin solution is much more pronounced. For both data sets, the base-line infection rate of MDCK cells remains unaffected by the amount of NaCl added.



**Figure 7.** Schematic representation of a possible mechanism that might allow mucin solutions to act as a barrier towards viral infection. Mucin biopolymers may offer competing binding sites that trap the viruses inside the biopolymer matrix (see main text for discussion). As a consequence, those viruses are prevented from reaching the epithelial surface.

**Table 1**

Survival of HeLa, A549 and MDCK-SIAT-1-CMV-PB1 cells after exposure to different mucin solutions and percentage of GFP-positive cells after HPV-16 inoculation in presence of mucins. ++ := > 85 %. -- := < 5 %. N.D.: not determined.

	buffer	1 % mucin	1 % Sigma mucin	1 % NBS mucin
viable HeLa cells	++	++	++	--
viable A549 cells	++	++	N.D.	N.D.
viable MDCK cells	++	++	N.D.	N.D.
GFP-positive HeLa cells after HPV-16 inoculation	(89.5 ± 0.1) %	(3.5 ± 1.5) %	(15.5 ± 0.7) %	N.D.