

Toward a Radically Simple Multi-Modal Nasal Spray for Preventing Respiratory Infections

John Joseph, Helna Mary Baby, Joselyn Rojas Quintero, Devin Kenney, Yohannes A Mebratu, Eshant Bhatia, Purna Shah, Kabir Swain, Dongtak Lee, Shahdeep Kaur, Xiang-Ling Li, John Mwangi, Olivia Snapper, Remya Nair, Eli Agus, Sruthi Ranganathan, Julian Kage, Jingjing Gao, James N Luo, Anthony Yu, Dongsung Park, Florian Douam, Yohannes Tesfaigzi,* Jeffrey M Karp,* and Nitin Joshi*

Nasal sprays for pre-exposure prophylaxis against respiratory infections show limited protection (20–70%), largely due to their single mechanism of action—either neutralizing pathogens or blocking their entry at the nasal lining, and a failure to maximize the capture of respiratory droplets, allowing them to potentially rebound and reach deeper airways. This report introduces the Pathogen Capture and Neutralizing Spray (PCANS), which utilizes a multi-modal approach to enhance efficacy. PCANS coats the nasal cavity, capturing large respiratory droplets from the air, and serving as a physical barrier against a broad spectrum of viruses and bacteria, while rapidly neutralizing them with over 99.99% effectiveness. The formulation consists of excipients identified from the FDA's Inactive Ingredient Database and Generally Recognized as Safe list to maximize efficacy for each step in the multi-modal approach. PCANS demonstrates nasal retention for up to 8 hours in mice. In a severe Influenza A mouse model, a single pre-exposure dose of PCANS leads to a >99.99% reduction in lung viral titer and ensures 100% survival, compared to 0% in the control group. PCANS suppresses pathological manifestations and offers protection for at least 4 hours. This data suggest PCANS as a promising daily-use prophylactic against respiratory infections.

1. Introduction

Respiratory infections result in significant morbidity and mortality worldwide.^[1] The past few decades have witnessed numerous outbreaks, often leading to epidemics or unanticipated pandemics such as COVID-19. Although vaccines are available against Influenza A virus (IAV), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), respiratory syncytial virus (RSV) and *Streptococcus pneumoniae*, the emergence of mutants often reduces the efficacy of vaccines.^[2] Additionally, there are several pathogens, including adenovirus, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Escherichia coli*, which can cause severe respiratory diseases, but do not have clinically available vaccines, as of now. In the face of an unforeseen pandemic, the timeline for developing vaccines targeting a pathogen can range from 1 to 10 years, contingent upon the specific nature of the pathogen.^[3,4]

J. Joseph, H. M. Baby, P. Shah, K. Swain, D. Lee, S. Kaur, X.-L. Li, J. Mwangi, O. Snapper, E. Agus, S. Ranganathan, J. Kage, J. Gao, A. Yu, J. M Karp, N. Joshi

Center for Accelerated Medical Innovation

Department of Anesthesiology

Perioperative and Pain Medicine

Brigham and Women's Hospital

Boston, MA 02115, USA

E-mail: jmkarp@bwh.harvard.edu; njoshi@bwh.harvard.edu

J. Joseph, H. M. Baby, P. Shah, D. Lee, S. Kaur, X.-L. Li, J. Mwangi, O. Snapper, E. Agus, S. Ranganathan, J. Kage, J. Gao, A. Yu, J. M Karp, N. Joshi

Center for Nanomedicine

Department of Anesthesiology

Perioperative and Pain Medicine

Brigham and Women's Hospital

Boston, MA 02115, USA

J. Joseph, J. R. Quintero, Y. A Mebratu, D. Lee, S. Kaur, R. Nair, J. Gao, J. N Luo, A. Yu, Y. Tesfaigzi, J. M Karp, N. Joshi

Harvard Medical School

Boston, MA 02115, USA

E-mail: ytesfaigzi@bwh.harvard.edu

J. R. Quintero, Y. A Mebratu, Y. Tesfaigzi

Division of Pulmonology

Brigham and Women's Hospital

Boston, MA 02115, USA

D. Kenney, F. Douam

National Emerging Infectious Diseases Laboratories

Department of Microbiology

Boston University

Chobanian & Avedisian School of Medicine

Boston, MA 02118, USA

E. Bhatia

Indian Institute of Technology

Mumbai 400076, India



The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/adma.202406348>

DOI: 10.1002/adma.202406348

The rapid creation of efficacious COVID-19 vaccines stands as an unparalleled scientific achievement. However, it took several months for the vaccine to become available, during which numerous hospitalizations and deaths were reported.^[5] Additionally, multiple obstacles, such as production complexities, vaccine nationalism, and the emergence of novel variants, collectively posed major challenges around the world. A large percentage of the population did not consent to vaccination for various reasons, which posed a significant hurdle in minimizing the transmission of pathogens. Another concern pertaining to vaccines is their partial mitigation of the pathogen burden,^[6,7] which implies that vaccinated people can still contract and disseminate the infection, albeit at a reduced rate compared to those who are unvaccinated. Thus, there is a critical need to develop a pre-exposure prophylactic approach that can be easily and rapidly deployed either independently or in tandem with vaccines, serving as the primary safeguard against current and emerging respiratory pathogens. Such an approach should efficiently reduce pathogen load, and be radically simple to scale up and manufacture to ensure widespread global adoption.

Transmission of most respiratory pathogens predominantly occurs through inhalation of contaminated respiratory droplets and their subsequent deposition in the nasal cavity, which has an entry checkpoint.^[8] For instance, SARS-CoV-2 virus binds to the angiotensin-converting enzyme 2 (ACE2) located in nasal epithelial cells via its receptor-binding domain (RBD). The nasal cavity is a primary target for SARS-CoV-2 infection due to high expression of ACE2,^[9–11] which decreases towards the lower respiratory tract.^[12] The infection spreads to the deeper airways via virus-laden extracellular vesicles secreted by infected cells in the nasal cavity.^[13] Similarly, bacteria, including *S. pneumoniae* and *S. aureus* adhere to nasal mucin via a specific adhesin receptor.^[14,15] Considering the vulnerability of nasal cavity and its critical role in the transmission of respiratory pathogens, chemoprophylactic nasal sprays have been developed to offer pre-exposure prophylaxis against respiratory infections. This approach utilizes chemical agents, including small molecule drugs, antiseptics, or nitric oxide, to deactivate the pathogen in the nasal cavity or a polymer that acts as a physical barrier to prevent pathogen entry through

the nasal lining.^[16,17] Although multiple pre-exposure chemoprophylactic approaches have been previously developed,^[18–20] they have resulted in sub-optimal efficacy with only 20–60% protection achieved in pre-clinical and clinical studies.^[21–23] We contend that the sub-optimal clinical efficacy of previous chemoprophylactic nasal sprays can be attributed, at least in part, to their dependence on a single mode of action, typically centered around either pathogen neutralization or hindering pathogen entry through the nasal lining. Additionally, as pathogens are deposited in the nasal cavity through the impaction of large respiratory droplets,^[24] the potential for these droplets to bounce off the cavity wall and reach the deeper airways has been overlooked in previous approaches. A comprehensive prophylactic strategy should prioritize the effective capture of pathogen-laden droplets, preventing them from bouncing off—a critical consideration neglected in prior efforts. Previous chemoprophylactic approaches also target a limited type/class of pathogen,^[21,25–28] which could potentially compromise their effectiveness against newly emerging pathogens. Finally, many chemoprophylactic nasal sprays face limitations for repeated/daily application due to toxicity concerns.^[29,30]

Herein, we report a Pathogen Capture and Neutralizing Spray (PCANS), which, unlike previously developed chemoprophylactic approaches, acts via a multi-modal approach that involves three key steps (**Figure 1**). First, PCANS enhances the capture of pathogen-laden respiratory droplets from inspired air by preventing them from bouncing off the nasal lining. Second, PCANS provides a physical barrier over nasal mucosa to intercept invasion/colonization of different pathogens. Last, PCANS rapidly neutralizes a wide range of pathogens. To ensure safety during daily or repeated use and achieve design simplicity, PCANS was meticulously designed as a “drug-free” formulation, utilizing materials from inactive ingredient database (IID) or generally recognized as safe (GRAS) list of the Food and Drug Administration (FDA) that are present as excipients in commercially available nasal/topical formulations and are available commercially in tons of quantities. Using a highly iterative approach, we performed rigorous screening of these excipients, their different concentrations and combinations to identify optimal agents and their concentrations that maximize the efficacy of each step of the multi-modal approach, while maximizing the residence time of PCANS in the nasal cavity. Consequently, the final formulation of PCANS comprises a blend of multiple active agents, each contributing to the overall effectiveness through a synergistic multi-modal approach. Given the diverse active agents in PCANS, we also conducted comprehensive studies to ascertain the optimal concentration for each agent, ensuring that their individual activities remained unaltered. This meticulous investigation was imperative to guarantee the efficacy at every stage of the multi-modal approach.

In vitro, PCANS demonstrated excellent physical barrier property against multiple viruses and bacteria preventing their transport by >99.99%. The unique composition of PCANS enabled broad-spectrum neutralization activity against a range of viruses (both enveloped and non-enveloped) and bacteria, resulting in >99.99% reduction in the pathogen load. On the other hand, iota/kappa carrageenan, the key component of a commercially available chemoprophylactic nasal spray- AGOVIRAX, resulted in only 90% reduction in the viral loads of pathogens includ-

J. N. Luo
Department of Surgery
Brigham and Women's Hospital
Boston, MA 02115, USA
D. Park
Center for Functional Nanomaterials
Brookhaven National Laboratory
Upton, NY 11973, USA
J. M. Karp
Harvard–Massachusetts Institute of Technology Division of Health
Sciences and Technology
Massachusetts Institute of Technology
Cambridge, MA 02139, USA
J. M. Karp
Broad Institute
Cambridge, MA 02142, USA
J. M. Karp
Harvard Stem Cell Institute
Cambridge, MA 02138, USA

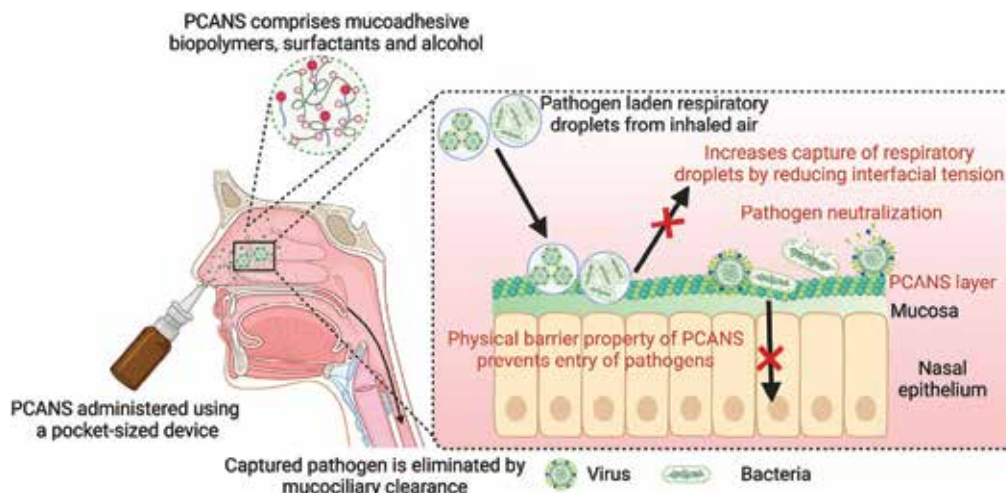


Figure 1. Pathogen Capture and Neutralizing Spray (PCANS) acts via a multi-modal approach against respiratory pathogens. An aqueous, “drug-free” solution of PCANS, comprising mucoadhesive biopolymers, surfactants, and alcohol, is administered using a pocket-sized nasal spray device and undergoes a phase transition to form a hydrogel layer over nasal mucosa. Surfactants in PCANS reduce interfacial tension of the nasal lining and increase wettability to enhance the capture or reduce the bounce-off of pathogen-laden respiratory droplets from the inhaled air. PCANS layers as a physical barrier preventing the transport of pathogens through the nasal lining. Finally, pathogens are neutralized by biopolymers and surfactants present in PCANS. PCANS is cleared via the native mucosal clearance mechanism and is eliminated through the digestive route.

ing IAV and SARS-CoV-2 viruses. Coating a 3D-model of human nasal cavity with PCANS significantly increased the capture of large respiratory droplets, compared to an only mucus-coated nasal cavity. Intranasal administration of PCANS-loaded with a fluorescent dye resulted in at least 8 h of residence time in the mouse nasal cavity, measured as the retention of fluorescence signal over time. No discernible tissue inflammation was observed in mouse nasal turbinate after repeated dosing of PCANS, establishing its safety in mice. In a proof-of-concept in vivo study performed in a mouse model of severe Influenza A infection induced by a supra-lethal dose of PR8 virus (a mouse-adapted H1N1 Influenza virus), PCANS showed superior prophylactic effect compared to the efficacy reported for previous approaches.^[21] Pre-exposure prophylactic administration of a single dose of PCANS was effective within 15 min, resulting in >99.99% reduction in lung viral titer, and 100% survival by day 10 as compared to 0% observed in the PBS-treated group. PCANS also suppressed pathological manifestations, and offered protection for at least 4 h. Overall, PCANS holds promise as a pre-exposure prophylactic approach to prevent current and emerging respiratory infections. The straightforward and readily scalable manufacturing process of PCANS, combined with its “drug-free” composition and robust stability demonstrated in this study, positions it favorably for widespread adoption and global distribution. To our knowledge, this is the first study to describe a multi-modal chemoprophylactic nasal spray, as well as the first to demonstrate broad-spectrum activity of a chemoprophylactic nasal spray against both bacteria and viruses. Additionally, this nasal spray is the first to achieve an 8-hour residence time in mouse nasal cavity, and the first chemoprophylactic strategy to show 100% protection in animals within a pre-clinical model of respiratory virus infection.

2. Results

2.1. Leveraging Biopolymers to Restrict Pathogen Entry via Formation of a Physical Barrier

We selected mucoadhesive biopolymers that are listed in the IID or GRAS list of the FDA and are present as excipients in commercially available nasal/topical formulations. Specifically, gellan, pectin, hydroxypropyl methylcellulose (HPMC), carboxymethyl cellulose sodium salt (CMC), carbopol, and xanthan gum were selected. The biopolymers were screened for their ability to impart physical barrier property to PCANS. Since a metered spray device would be used to administer PCANS, we first identified sprayable concentration of each biopolymer by performing rheological measurements (Figure 2a–f). Dynamic viscosity curves were generated using a rotational rheometer by varying shear rates up to 40 s⁻¹, which is within the lower limits of shear rates encountered while dispensing formulations through a nasal spray device. Concentrations that exhibited a viscosity of less than 0.1 Pa.s were considered “sprayable”.^[31,32] Next, we determined the mechanical strength of each biopolymer at the highest sprayable concentration before and after the addition of simulated nasal fluid (SNF). SNF was added to mimic the physiological environment in the nasal cavity. Mechanical strength was measured using a rotational rheometer and quantified as storage modulus (G'), which represents the amount of structure present in a material.^[33] In the presence of SNF, gellan showed the highest G' as compared to other biopolymers (Figure 2g), indicating its superior mechanical strength. Gellan showed a 100-fold increase in its G' in the presence of SNF (Figure 2g), which is consistent with its ability to undergo in situ gelation under physiological conditions. Mono and divalent cations present in the

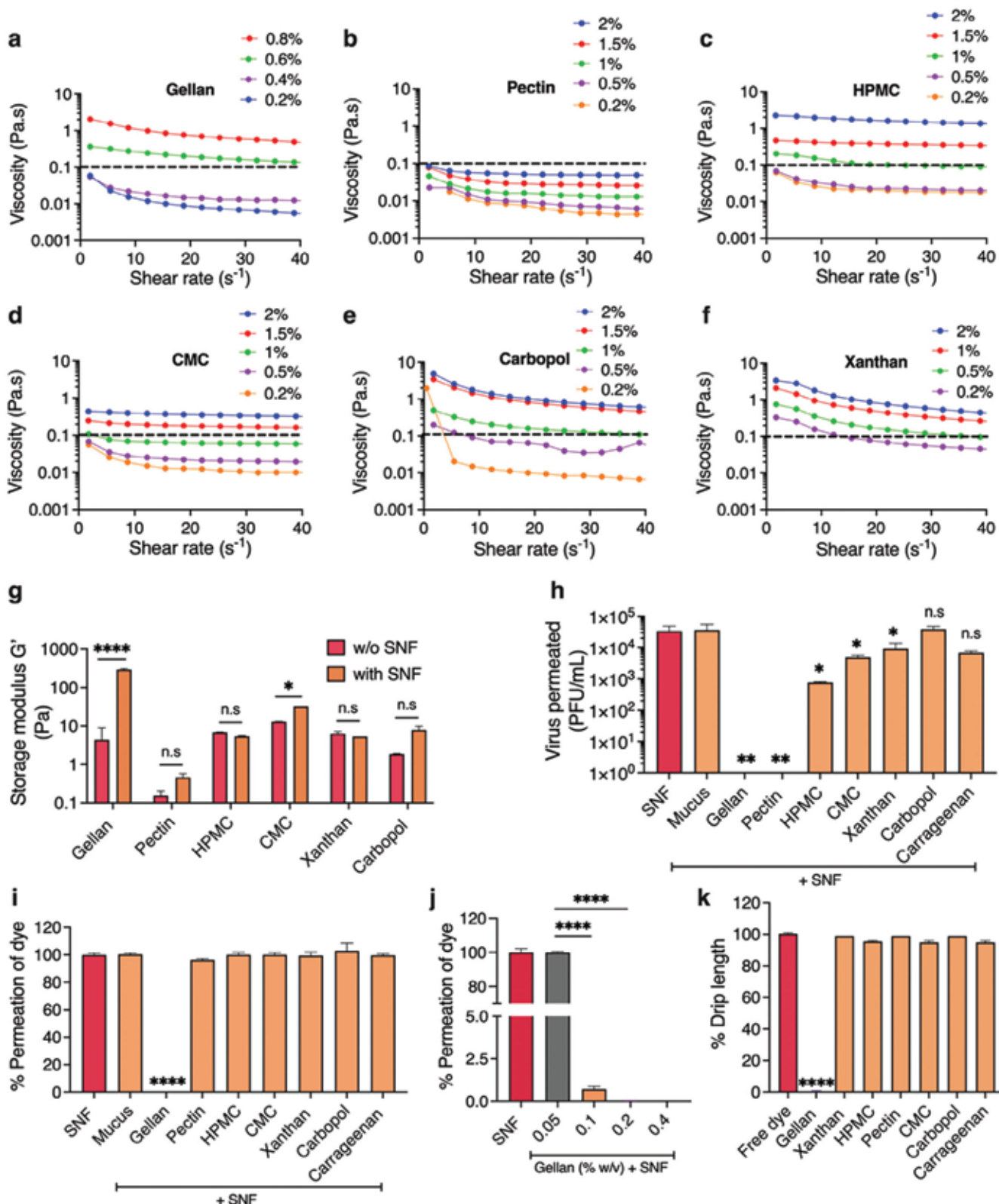


Figure 2. Biopolymers were screened for physical barrier property against pathogen entry. Viscosity as a function of shear rate up to $40\ s^{-1}$ at $25\ ^\circ C$ for different concentrations of a) gellan, b) pectin, c) hydroxy propyl methyl cellulose (HPMC), d) carboxymethylcellulose (CMC), e) Carbopol and f) xanthan gum in water. The sprayable viscosity window is shown below the dashed line. g) Storage modulus (G') of 0.4% (w/v) gellan, 2% (w/v) pectin, 0.5% (w/v) HPMC, 0.5% (w/v) CMC, 0.2% (w/v) Carbopol, and 0.2% (w/v) xanthan gum, without and with simulated nasal fluid (SNF). Amplitude sweep

SNF complex with glucuronic monomeric units of gellan to form a crosslinked hydrogel.^[34] Compared to gellan, other biopolymers showed minimal or no increase in their storage modulus, suggesting poor in situ gelation. To investigate physical barrier property of biopolymers, a trans-membrane assay was devised (Figure S1, Supporting Information), which involved evaluating the transport of IAV through an SNF-coated cell strainer (pore size $\approx 70\ \mu\text{m}$) or a cell strainer coated with simulated mucus/SNF mixture or a biopolymer/SNF mixture. After 4 h, the viral titer in the chamber below the strainer was quantified by performing a plaque assay in Madin-Darby canine kidney (MDCK) host cells. Consistent with its excellent mechanical strength, Gellan/SNF reduced the transport of IAV particles by >4 -log fold (99.99%) as compared to only SNF-coated or mucus/SNF-coated strainers (Figure 2h). Xanthan/SNF, CMC/SNF and HPMC/SNF also significantly reduced the IAV transport, but not as efficiently as gellan/SNF. Interestingly, despite significantly lower mechanical strength of pectin/SNF as compared to gellan/SNF, it intercepted the IAV transport with similar efficiency as gellan/SNF. Carrageenan, a biopolymer used in previously reported and commercially available chemoprophylactic nasal sprays,^[35] was used as a control and did not reduce IAV transport in the presence of SNF.

Reduction in the transport of IAV particles by anionic biopolymers could be a result of their physical barrier property and/or electrostatic interactions between their negatively charged polymeric chains and the positively charged capsid of IAV. To decouple the effects of physical barrier property and electrostatic interactions, we studied the transport of a low molecular weight dye, rhodamine B isothiocyanate, which does not exhibit electrostatic interactions with any of the biopolymers evaluated, as confirmed using an in silico study (Figure S2, Supporting Information). Gellan/SNF resulted in 100% reduction in the transport of the dye, confirming excellent physical barrier property (Figure 2i). In contrast, other biopolymers did not reduce the transport of the dye, indicating their poor physical barrier property. This confirms that the reduction in IAV transport by pectin was primarily mediated via electrostatic interaction of pectin's chains with the virus capsid.

Next, we aimed to investigate the underlying mechanism responsible for the observed reduction in the transport of rhodamine B isothiocyanate by the gellan/SNF system. Data from the in silico study exploring the intermolecular interactions of rhodamine B isothiocyanate with different biopolymers (Figure S2 and Table S1, Supporting Information) indicated that the dye interacts with gellan primarily through hydrogen bonds, similar

to its interactions with other biopolymers. Notably, rhodamine B isothiocyanate does not display hydrophobic interactions with gellan but does with certain other biopolymers. Therefore, the interactions between the dye and gellan do not appear to be unique and are unlikely to be the primary factor contributing to the observed inhibition of the dye transport with gellan/SNF. Interestingly, gellan/SNF reduced the transport of rhodamine B isothiocyanate in a concentration-dependent manner (Figure 2j), with a minimum of 0.1% w/v gellan required. Higher polymer concentration in a gel can increase tortuosity.^[36] As the concentration of the polymer increases, the network of cross-linked polymer chains becomes denser. This increased tortuosity creates more complex and convoluted diffusion pathways, thereby reducing the effective diffusion coefficient. Previous studies have shown that in systems with large pores, where the pore diameter is approximately 30–40 times the size of the diffusing molecule, geometric tortuosity is the primary factor influencing diffusive transport.^[37] In contrast, for pores with diameters barely 10 times the molecular size, intermolecular interactions are more pronounced. Given the small size of rhodamine B isothiocyanate ($\approx 2\ \text{nm}$) relative to the micron-sized pores in the gellan gel,^[38] the diffusion of the dye through gellan gel aligns with the former scenario, suggesting that the observed transport reduction could be attributed to the increased tortuosity caused by the gel's cross-linking. Another physical factor responsible for the reduced transport of the dye could be the mechanical strength of the gel, measured as the storage modulus (G'), which increased with the concentration of gellan (Figure S3, Supporting Information). An increase in the mechanical strength of gels results in reduced swelling and hence reduces the diffusivity of molecules.^[39] Therefore, taken together, the primary drivers for the reduction in dye transport by the gellan/SNF can be attributed to both increased tortuosity and gel strength at specific gellan concentrations.

Gellan/SNF also showed excellent physical barrier property against bacteria. A 0.2% w/v concentration of gellan also reduced the transport of *E. coli* bacteria by >8 -log fold (100%) (Figure S4, Supporting Information), suggesting its broad spectrum physical barrier property to limit the transport of both viruses and bacteria. Mucus, on the other hand, only showed a 1-log fold (90%) reduction. To conclude, gellan at a concentration of 0.2% w/v and above impeded the transport of rhodamine B dye, *E. coli*, and IAV by 100%.

To ensure maximum coverage of the nasal cavity, we evaluated the spray characteristics of gellan at a concentration of 0.2% w/v or higher when sprayed using a nasal spray pump (Aptar).

measurements were performed at 37 °C by varying oscillatory strain between 0.005% to 10% at 1 Hz frequency. **** $P < 0.0001$, * $P < 0.05$. n.s., not significant. h) Amount of Influenza A virus (IAV) that permeated within 4 h through a simulated nasal fluid (SNF)-coated cell strainer (pore size $\approx 70\ \mu\text{m}$) or a cell strainer coated with simulated mucus/SNF mixture or a biopolymer/SNF mixture. Permeation of viral particles was quantified by evaluating the viral titer in the chamber below the strainer using plaque assay performed in MDCK host cells. Results are expressed in plaque-forming units (PFU/mL). ** $P < 0.01$, * $P < 0.05$ compared to mucus/SNF, n.s., not significant. Percentage permeation of a fluorescent dye, rhodamine B isothiocyanate through i) an SNF-coated cell strainer or a cell strainer coated with simulated mucus/SNF mixture or a biopolymer/SNF mixture. **** $P < 0.0001$ compared to mucus/SNF and j) an SNF-coated strainer or strainer coated with gellan/SNF at different concentrations of gellan. **** $P < 0.0001$ compared to 0.05% w/v gellan/SNF. k) Percentage drip length of free brilliant green dye or mucoadhesive polymers mixed with brilliant green dye on sheep mucosal tissue. Drip length from the spray area was measured as the distance traversed in 4 h by the biopolymer or free dye from the point of deposition. The percentage drip length of each biopolymer was calculated with respect to the drip length of the free dye. **** $P < 0.0001$ compared to free dye. For g and h, P -values were determined using two-way ANOVA with Tukey's multiple comparisons tests. For i-k, P -values were determined using one-way ANOVA with Tukey's post hoc analysis. Data in a-f are from a single experiment (experiment repeated three times). Data in g-k are means $\pm$ SD of technical repeats ($n = 3$, each experiment performed at least twice).

Plume geometry and spray coverage were measured with a high-speed image acquisition system. Increasing gellan concentration resulted in a significant reduction in the angle of emitted plume of the spray (defined as “plume angle”) and coverage area (Figure S5a–d, Supporting Information). Henceforth, we used 0.2% w/v gellan due to its superior physical barrier property, plume angle, and coverage area, as compared to other concentrations.

Next, we evaluated the retention ability of gellan and other biopolymers at the mucosal tissue upon spraying. Mucosal retention was measured as the drip length, defined as the distance traversed in 4 h by the biopolymer from the point of deposition on sheep’s intestinal mucosa placed vertically. To visualize dripping, biopolymers were mixed with a brilliant green dye. The percentage drip length of each biopolymer was calculated with respect to the drip length of the free dye. Gellan (0.2% w/v) demonstrated excellent mucosal retention with zero drip length (Figure 2k; Figure S6, Supporting Information). Other biopolymers, including carrageenan, which was used as a control showed >95% drip length, indicating poor mucosal retention. Gellan’s superior mucosal retention is attributed to its ability to strongly entangle with mucin glycoprotein in the mucosal tissue during the sol-gel transition.^[40]

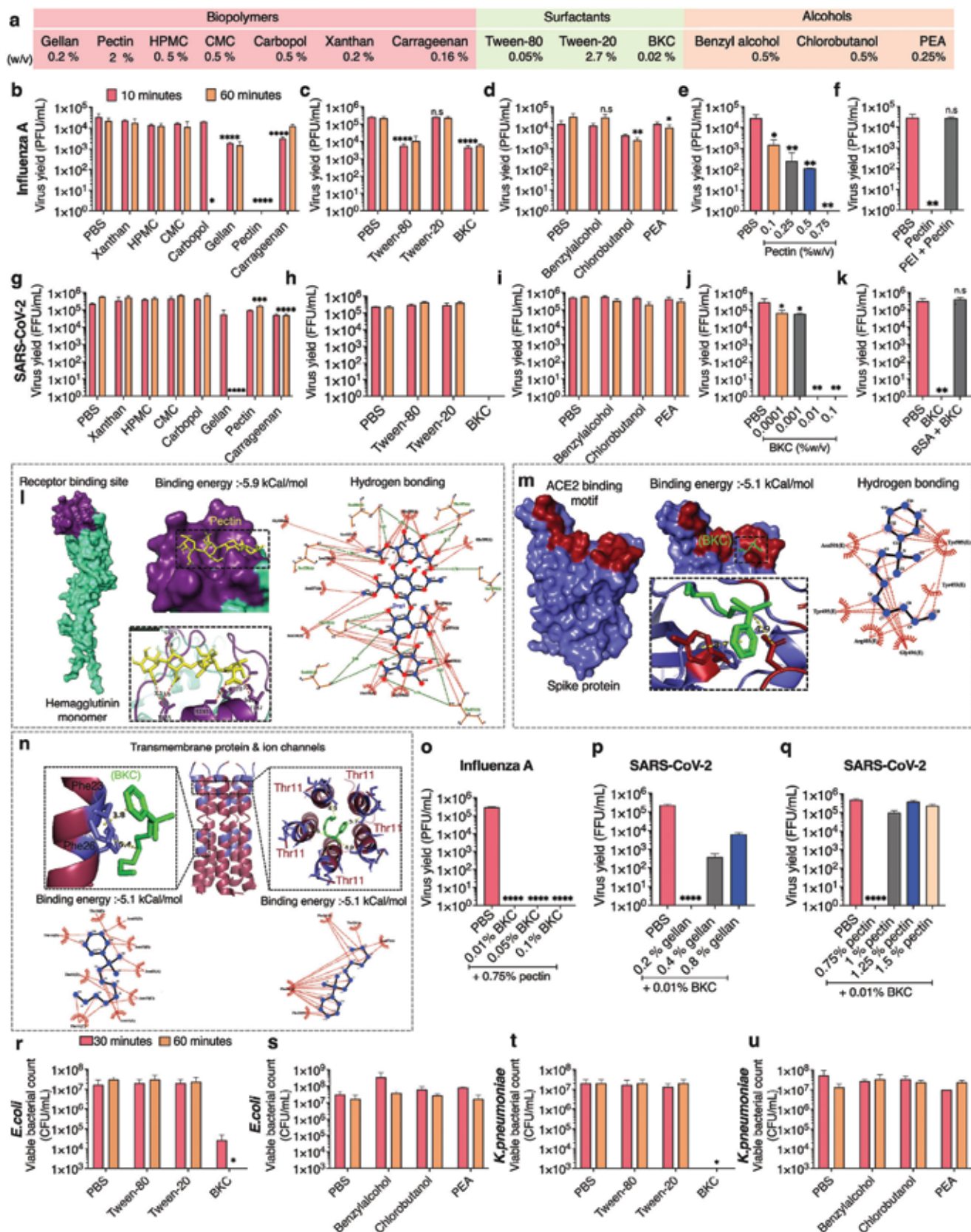
2.2. Identifying Agents for Neutralizing a Broad-Spectrum of Respiratory Pathogens

To impart PCANS a broad-spectrum pathogen neutralization ability, we screened agents from three different classes of compounds, including biopolymers, surfactants, and alcohols. We define “neutralization” as a process that impedes pathogen entry into host cells by either destabilizing the pathogen cell membrane or blocking the receptor-mediated binding/fusion of the pathogen through chemical interactions. These compounds were selected based on their previously reported ability to neutralize different types of pathogens.^[41–43] To maximize safety and translatability of PCANS, we only selected agents that are listed in the IID or GRAS list of the FDA and are present as excipients in commercially available nasal/topical formulations (Figure 3a). We first evaluated the neutralization ability of these agents against viruses (Figure 3b–k). Neutralization was studied *in vitro* by incubating each agent individually with either IAV or SARS-CoV-2 for 10 or 60 min, followed by 1-min centrifugation and subsequent infection of target cells with the supernatant evaluated using plaque forming or focus-forming assay. We chose IAV and SARS-CoV-2 due to their high prevalence worldwide as respiratory viruses and also due to a difference in their capsid proteins and charge.^[44,45] Biopolymers were evaluated at their highest sprayable concentration, except for gellan and carrageenan. Gellan was evaluated at 0.2% w/v due to its superior physical barrier property compared to 0.1% w/v concentration and superior spray pattern compared to 0.4% w/v concentration. Carrageenan, used as a control, was evaluated at 0.16% w/v, as this concentration is present in a commercially available chemoprophylactic nasal spray.^[46,47] Surfactants and alcohols were evaluated at the highest concentration previously used in humans via nasal route.^[48,49] Compared to carrageenan, pectin exhibited superior neutralization of IAV, regardless of the incubation time, and demonstrated a 4-log fold (99.99%) reduction in viral titer in

the host cells in comparison to PBS (Figure 3b). Ten min of incubation with carbopol did not reduce the IAV titer, but a 4-log fold (99.99%) reduction was observed with 60 min of incubation. Gellan exhibited similar neutralization of IAV as carrageenan, resulting in only a 1-log fold (90%) reduction in viral load in the host cells. For SARS-CoV-2, both pectin and carrageenan showed less than a 1-log fold decrease in viral load in the host cells (Figure 3g). Gellan showed a 4-log fold (99.99%) reduction in the viral titer, but only with 1 h incubation time. Among surfactants, tween 80 and benzalkonium chloride (BKC) showed a 1-log log fold reduction in IAV titer in the host cells, regardless of the incubation time (Figure 3c). Rapid neutralization of SARS-CoV-2 was observed with BKC, resulting in a 5-log fold (>99.99%) reduction in viral load in the host (Figure 3h). Alcohols did not neutralize SARS-CoV-2, and minimum neutralization was observed for IAV, resulting in less than 1-log fold (90%) reduction in viral load for chlorobutanol and phenethyl alcohol (PEA) (Figure 3d,i). Overall, this extensive screening identified pectin and BKC as the most effective agents for rapid neutralization of IAV and SARS-CoV-2, respectively. Neutralization ability of pectin and BKC was found to be dose-dependent (Figure 3e,j). Minimum concentrations of 0.75% w/v and 0.01% w/v were required for pectin and BKC, respectively, to achieve >4-log fold (>99.99%) reduction in the viral load with 10 min of incubation time.

To elucidate the viral neutralization mechanism of pectin and BKC, we performed *in silico* modeling to determine their binding affinity with the receptor binding domains (RBD) of IAV and SARS-CoV-2, respectively. For IAV, anionic pectin targets RBD at the distal part of hemagglutinin, which is positively charged, thus averting the virus entry into the host cell (Figure 3l). Compared to the host ligand sialic acid present in mucosal epithelia, pectin showed stronger binding to RBD through distant hydrogen bonding with Ser228, Ser186, and Thr187 and hydrophobic linkage with Ser227 and Glu190 (Figure S7, Supporting Information). BKC was found to exhibit hydrophobic interactions with the ACE2 binding motif of spike protein of SARS-CoV-2 (Figure 3m). BKC also showed hydrophobic interactions with Phe23 and Phe26 in membrane helices via pi-pi stacking (Figure 3m), which can distort the helical conformation of adjacent helices, as aromatic stacking of Phe23 and Phe26 is a prerequisite to stabilizing helix-helix interface of the envelope transmembrane protein. BKC fits into the pentameric ion channels at the N terminus of the transmembrane domain through interaction with Thr11 and potentially blocks the influx/efflux of ions (Figure 3n). To determine the role of electrostatic interaction in pectin- and BKC-mediated neutralization of IAV and SARS-CoV-2, respectively, we performed a neutralization assay by pretreating pectin and BKC with counter ions to offset the charge. As anticipated, anionic pectin in the presence of positively charged polyethyleneimine lost its neutralization activity and failed to show a significant reduction in the viral load compared to PBS (Figure 3f). Likewise, the pretreatment of BKC with negatively charged bovine serum albumin diminished the ability of BKC to reduce the SARS-CoV-2 titer in the host cells (Figure 3k).

Next, we investigated whether ionic interactions between anionic gellan or pectin with cationic BKC, when present together in a formulation, would impact the neutralization ability of pectin or BKC. Notably, the neutralization efficiency of pectin (0.75% w/v) against IAV remained conserved even with a



dose-dependent increase in BKC up to a concentration of 0.1% w/v (Figure 3o). Neutralization efficiency of BKC (0.01% w/v) against SARS-CoV-2 was not impacted by gellan or pectin at 0.2% w/v or 0.75% w/v concentrations, respectively, but reduced at higher concentrations (Figure 3p,q). These results further underscore that the concentration of each agent is critical for efficient neutralization.

Finally, we also screened surfactants and alcohols to assess their neutralization ability against bacteria, including *E. coli* and *K. pneumoniae*. Neutralization was determined by measuring the bactericidal activity. Each agent was individually incubated with either *E. coli* or *K. pneumoniae* for 30 or 60 min, followed by 1-min centrifugation, and then evaluating the bacterial load in the supernatant using a colony-forming assay. BKC was more effective than non-ionic surfactants, resulting in a 4-log fold (99.99%) and 7-log fold (99.99%) reduction in colony-forming units (CFU) of *E. coli* and *K. pneumoniae*, respectively, with an incubation time of 30 min (Figure 3r,t). Previously reported molecular modeling studies have shown that BKC incorporates into the bacterial membrane, resulting in its destabilization.^[50] This involves BKC's attachment to the bacterial cell surface followed by its quick integration into the lipid bilayer, where it stays for at least several nanoseconds, likely causing membrane destabilization. Alcohols had a negligible bactericidal effect over the exposure periods of 30 or 60 min (Figure 3s,u). Altogether, our data on physical barrier property, spray pattern, mucosal retention, and neutralization indicate gellan, pectin, and BKC as the three critical components to formulate PCANS. However, we also incorporated phenethyl alcohol (PEA), as it is commonly added as a stabilizer to nasal formulations to prevent the growth of gram-negative bacteria,^[48] thereby ensuring a long shelf-life.

2.3. Utilizing Surfactants to Promote the Capture of Respiratory Droplets

Pulmonary surfactant layers the alveolar epithelium to enhance wettability and trap airborne particles.^[51] We adopted this biomimetic approach to capture pathogen-laden respiratory droplets in the nasal cavity. Specifically, we identified surfactants

to reduce interfacial tension of PCANS and reduce the bounce off/escape of respiratory droplets. We evaluated surfactants listed in the IID list, including Tween-20, Tween-80, and BKC. Screening was performed using a twin impinger, which is a glass apparatus that can be used to assess the deposition of aerosolized particles in different regions of the respiratory tract (Figure 4a).^[52,53] Simulated mucus or a biopolymer mixture of gellan (0.2% w/v) and pectin (0.75% w/v) without or with different concentrations of surfactants was sprayed into the SNF-coated oropharyngeal region of the impinger (Figure 4a). Droplets with mass medial aerodynamic diameter >5 μm and laden with rhodamine B-loaded liposomes (size ≈ 400 nm) were generated using a jet nebulizer to mimic pathogen-laden large respiratory droplets. Droplet capture was determined by quantifying the fluorescence intensity of rhodamine B in the biopolymer/surfactant mixture or the mucus layer. Biopolymer mixture without any surfactant showed similar fluorescence intensity as mucus (Figure 4b). Combining the biopolymer mixture with Tween-80 or Tween-20 at a concentration higher than 0.005% w/v or with BKC at a concentration higher than 0.01% w/v resulted in a significant increase in the fluorescence intensity as compared to mucus or only biopolymer mixture, suggesting increased capture of droplets due to surfactants. Compared to Tween-20, BKC and Tween-80 resulted in a significantly higher fold increase in the fluorescence intensity when added to the biopolymer mixture at a concentration of 0.05% w/v or higher (Figure 4b). At 0.05% w/v concentration, both BKC and Tween-80 containing biopolymer mixtures showed similar fluorescence intensity, which was 4-fold higher than the fluorescence intensity of mucus or biopolymer mixture without a surfactant. Since 0.01% w/v is the most commonly used concentration of BKC in commercially available nasal formulations,^[54,55] and also showed excellent neutralization activity against SARS-CoV-2, we decided to use this concentration in PCANS, even though BKC didn't increase the capture of respiratory droplets at this concentration.

To impart respiratory droplet-capturing ability, we decided to proceed with Tween-80 and determined its safe concentration that would not compromise the permeability or metabolic activity of nasal epithelium. To that end, we performed an in vitro

Figure 3. Biopolymers, surfactants and alcohols were screened for neutralization of different respiratory pathogens. a) Table summarizes different components and their concentrations to determine the neutralization ability against respiratory pathogens. Each component was individually evaluated for its pathogen neutralization potential. IAV and SARS-CoV-2 viral loads in the host cells after 10 or 60 min incubation of the virus with b, g) different biopolymers, c, h) different surfactants, and d, i) different alcohols. Viable viral titer was quantified using plaque assay in MDCK host cells for IAV and focus-forming assay in Vero E6 cells for SARS-CoV-2 virus. Results are expressed in plaque-forming units (PFU/mL) or focus-forming units (FFU/mL). **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared to 10 minutes of incubation with PBS. n.s., not significant. Viral loads in the host cells after 10 min incubation of e) IAV and j) SARS-CoV-2 with different concentrations of pectin and BKC, respectively. ** $P < 0.01$, * $P < 0.05$ compared to PBS. Viral loads in the host cells after 10 min incubation of f) IAV and k) SARS-CoV-2 with pectin (0.75% w/v) + polyethylenimine and BKC (0.01% w/v) + bovine serum albumin, respectively. ** $P < 0.01$ compared to PBS. n.s., not significant. l) Pectin (yellow) binds to the receptor binding site of IAV (purple) at the distal part of hemagglutinin monomer (colored in purple) through hydrophobic interactions with Ser227 and Glu190, and hydrogen bonding with Ser228, Ser186, and Thr187. Blue and red dots in hydrogen bonding maps represent carbon and oxygen atoms, respectively. m) Chemical interaction of BKC (green) with ACE2 binding motif (red) in the spike protein of SARS-CoV-2. Interaction map reveals the hydrogen bonding of BKC with Tyr505 and Gly496. n) Aromatic pi-pi interaction of BKC (green) with Phe23 (purple) in the transmembrane domain and with Thr11 (brown) membrane helices. Interaction analysis shows 10 hydrophobic bonds with Phe23 and 8 hydrophobic bonds with Phe26. o) Viral load in host cells after 10 min incubation of IAV with pectin (0.75% w/v) in the presence of different concentrations of BKC. **** $P < 0.0001$ compared to PBS. Viral load in the host cells after 10 min incubation of SARS-CoV-2 with BKC (0.01% w/v) in the presence of different concentrations of p) gellan and q) pectin. **** $P < 0.0001$ compared to PBS. Effect of surfactants (r, t) and alcohols (s, u) against gram-negative bacteria *E. coli* and *K. pneumoniae* using colony-forming unit (CFU) plate count method after 30 and 60 minutes of exposure. Viable bacterial colonies are expressed in CFU/mL. * $P < 0.05$ compared to PBS. For b–d, P values were determined using two-way ANOVA with Tukey's post hoc analysis. For e, f, j, k, o–q, P values were determined using one-way ANOVA. Data in b–k and o–u are presented as Means $\pm$ S.D of technical repeats ($n = 3$, each experiment performed at least twice). Ser, serine; Thr, threonine; Glu, glutamic acid; Phe, phenylalanine; Tyr, tyrosine; Gly, glycine.

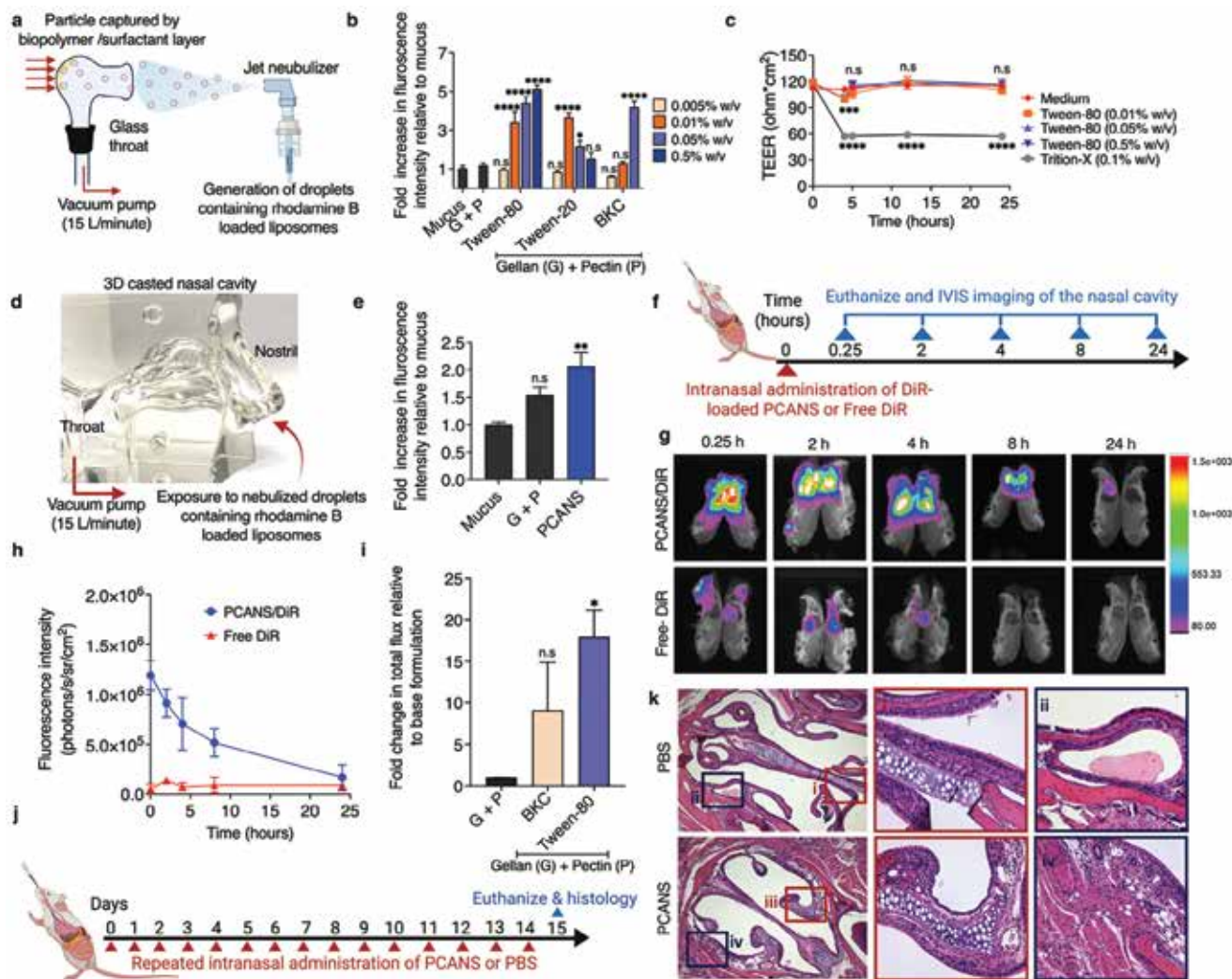


Figure 4. PCANS enhances the capture of respiratory droplet-mimicking aerosol and exhibits prolonged nasal residence time in mice. **a)** Experimental design for measuring the capture of respiratory droplet-mimicking aerosol. A twin impinger was used to simulate the aerodynamics of the human respiratory tract. Mucus or gellan (0.02% w/v) and pectin (0.75% w/v) solution (G+P), without or with different concentrations of Tween-80, Tween-20 or BKC was coated on the inner surface of the throat region of the impinger using a nasal spray device. Droplets with mass medial aerodynamic diameter $>5 \mu\text{m}$ and laden with rhodamine B-loaded liposomes (size $\approx 400 \text{ nm}$) were generated using a jet nebulizer and administered into the impinger under vacuum (15 L min^{-1}). Droplet capture was determined by quantifying the fluorescence intensity of rhodamine B in the biopolymer/surfactant mixture or mucus layer. **b)** Fold increase in fluorescence intensity with respect to mucus. $****P < 0.0001$, $*P < 0.05$ compared to mucus. **c)** Transepithelial electrical resistance (TEER) across the human nasal epithelial cell (RPMI-2650)-based monolayer at different time points after treatment with only medium or medium containing Triton-X (0.1% w/v) or different concentrations of Tween-80. Surfactant-containing medium was replaced at 4 h with fresh medium to examine impedance recovery. $****P < 0.0001$, $***P < 0.001$ compared to untreated control. n.s., not significant. **d)** Experimental design for measuring the capture of respiratory droplet-mimicking aerosol using a 3D human nasal cavity model (Koken cast). The inner surface of the nasal cavity was coated with mucus, G+P solution or PCANS (the final formulation) using a nasal spray device. The throat part of the model was coupled to a vacuum pump for simulating the respiratory airflow (15 L min^{-1}). Nostrils were then exposed to nebulized rhodamine B-loaded liposomes for 1 minute. Droplet capture was determined by quantifying the fluorescence intensity of rhodamine B in the nasal cavity. **e)** Fold increase in fluorescence intensity with respect to mucus. $**P < 0.01$ compared to mucus. n.s., not significant. **f)** Experimental outline for the evaluation of the nasal residence time of PCANS in mice. C57BL/6 mice were intranasally administered with $10 \mu\text{L}$ of free DiR or DiR-loaded PCANS (PCANS/DiR) into each nostril. Mice were euthanized at different time points over 24 h, and nasal cavity was harvested and imaged using an in vivo imaging system (IVIS). **g)** Representative images of the nasal cavity excised at different time points. **h)** Quantification of fluorescence intensity in the nasal cavity at different time points. **i)** Fold change in total flux at 8 h in the nasal cavity relative to G+P. $*P < 0.05$, compared to G+P. n.s., not significant. **j)** Experimental design to assess the biocompatibility of PCANS in mouse nasal cavity. $10 \mu\text{L}$ PCANS or PBS was administered into each nostril of C57BL/6 mice once daily for 14 consecutive days. Animals were euthanized on day 15 and nasal cavity was analyzed histologically. **k)** Representative images of H&E-stained sections of nasal turbinate from mice captured using a 4X objective. Insets represent healthy olfactory epithelium (i) and (iii), and lamina propria (ii) and (iv) captured at 20X objective. For **b**, **e**, and **i**, P values were determined by one-way ANOVA using Tukey's post hoc analysis. For **b**, concentrations for each surfactant were compared individually. For **c**, P values were determined by two-way ANOVA with Tukey's multiple comparison test. Data in **b**, **c**, and **e** are presented as Means $\pm$ S.D of biological repeats ($n = 3$, each experiment performed at least twice). Data in **h** and **i** are presented as Means $\pm$ SEM ($n = 5$ mice/group).

assay evaluating the transepithelial electrical resistance (TEER) across the human nasal epithelial cell (RPMI-2650)-based monolayer upon treatment with different concentrations of tween-80. A transient dip of less than 15% in TEER was observed in the monolayer immediately after the addition of Tween-80, irrespective of the concentrations evaluated in this study. However, the TEER reversed rapidly to the original value in less than 1 h after replacing tween-80-containing medium with fresh medium (Figure 4c). The drop in the TEER for Tween-80 was significantly less compared to Triton-X (negative control), which resulted in a permanent change in the TEER. Second, we evaluated the effect of different concentrations of Tween-80 on the metabolic activity of RPMI-2650 cells upon 24 or 48 h of incubation. Cells incubated with 0.01% or 0.05% w/v tween-80 showed similar metabolic activity as cells incubated in medium. However, Tween-80 (0.5% w/v) resulted in a significant reduction in the metabolic activity of RPMI cells (Figure S8, Supporting Information). Thus, we decided to use 0.05% w/v as the final concentration of Tween-80 in PCANS. Overall, based on our data for physical barrier property, spray pattern, mucosal retention, neutralization, droplet capture, and nasal epithelial cell toxicity, we decided on gellan, pectin, BKC, PEA, and Tween-80 as the final components for PCANS, and validated the respiratory droplet capturing ability of the final formulation using a 3D-model of human nasal cavity (Koken cast) with the anatomical intricacies (Figure 4d).^[56] Consistent with the twin impinger results, there was no significant difference in the fluorescence intensity between gellan and pectin mixture, and mucus (Figure 4e). PCANS, on the other hand, showed a 2-fold higher fluorescence compared to mucus, suggesting the potential of PCANS to increase the capture of pathogen-laden respiratory droplets from inhaled air.

2.4. Prolonged Nasal Retention of PCANS and Safety Upon Repeated Administration

Next, we evaluated the retention of PCANS in the nasal cavity of mice (Figure 4f). PCANS (10 μ L) mixed with a fluorescent dye – (DiI18(7) (1,1'-Diocadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide) (DiR) was administered into both nostrils of C57/BL6 mice. Free DiR was used as a control. Mice were euthanized at different time points over 24 h, and nasal cavity was harvested and imaged using an in vivo imaging system (IVIS) to quantify the fluorescence signal from DiR. Free DiR resulted in negligible fluorescence signal, even at 15 min after administration, suggesting its rapid clearance (Figure 4g,h). Interestingly, mice administered with DiR-loaded PCANS showed significant fluorescence for up to 8 h, suggesting prolonged nasal retention of PCANS (Figure 4g,h). We hypothesized that prolonged retention of PCANS is attributed to the presence of surfactants, including Tween-80 and BKC, which have previously been shown to reduce cilia beat frequency in the nasal cavity.^[57] To test our hypothesis, we compared nasal retention of DiR-loaded mixture of gellan and pectin without or with tween-80 or BKC. The addition of both BKC or tween-80 significantly enhanced the nasal retention of gellan and pectin mixture at 8 h post-nasal administration, as evident from the fluorescent signal of DiR in the nasal cavity (Figure 4i). However, tween-80

resulted in significantly higher nasal retention than BKC. The ability of tween-80 to enhance the nasal retention of gellan and pectin mixture was found to be concentration-dependent (Figure S9, Supporting Information). However, considering irreversible nasal epithelial permeabilization and cytotoxicity at 0.5% w/v or higher concentration of tween-80, we maintained 0.05% w/v in PCANS for further experiments. Notably, nasal administration of DiR-loaded PCANS only showed fluorescence signals in the nasal cavity and stomach, suggesting no systemic absorption. PCANS was fully cleared at 24 h (Figure 4g,h; Figure S10, Supporting Information), resulting in negligible fluorescence signal in both the nasal cavity and the stomach. To confirm safety of PCANS, we performed a repeat-dose toxicity study in healthy mice intranasally administered with PCANS or PBS once daily for 14 consecutive days (Figure 4j). Hematoxylin and eosin (H&E) stained sections of nasal cavity from both PBS or PCANS-administered mice did not show any inflammation or other gross evidence of toxicity, as evident by a defined lamina propria (Figure 4k). This connotes the safety of PCANS for daily administration.

2.5. Broad-Spectrum Activity, Spray Characteristics, and Shelf Stability of PCANS

Having identified the final components of PCANS, along with their optimal concentrations, we sought to demonstrate the physical barrier property and neutralization ability of PCANS against a broad spectrum of respiratory pathogens, including enveloped viruses (IAV, SARS-CoV-2, RSV), a non-enveloped virus (adenovirus), and bacteria (*E. coli* and *K. pneumoniae*). Physical barrier property was evaluated by assessing the transport of pathogens through an SNF-coated cell strainer or a cell strainer coated with simulated mucus/SNF mixture or PCANS/SNF mixture. PCANS/SNF prevented the transport of all the pathogens by >4-log fold (>99.99%) (Figure 5a-f), suggesting its broad-spectrum physical barrier property. For all pathogens, except RSV, mucus/SNF mixture showed significantly less prevention of pathogen transport compared to PCANS/SNF. PCANS also efficiently neutralized all the tested pathogens within 10 or 30 min of incubation time, resulting in >3-log fold (>99.9%) reduction in pathogen load (Figure 5g-l). Excitingly, PCANS demonstrated remarkable efficacy in co-neutralizing both bacterial and viral pathogens (Figure S11, Supporting Information), achieving >4-fold (>99.99%) reduction in the load of *E. coli* and SARS-CoV-2, used as proof-of-concept pathogens in this experiment. This finding is clinically significant, as respiratory droplets can carry both bacterial and viral pathogens, highlighting PCANS's potential in providing comprehensive protection against respiratory infections. We also evaluated the spray characteristics of PCANS sprayed through a standard and commercially used VP3 multi-dose nasal spray pump (Aptar, USA). The droplet distribution data showed that 10% of PCANS droplets had size >10 μ m, and 90% had size <200 μ m (Figure 5m), which is desirable to maximize the deposition in nasal cavity, while minimizing deposition into deep lungs. PCANS resulted in a wide plume angle within the ideal range of 35–55°, an ovality close to 1, covering a circular area of up to 8%, which is in line with the commercial nasal sprays (Figure 5m).^[58,59]

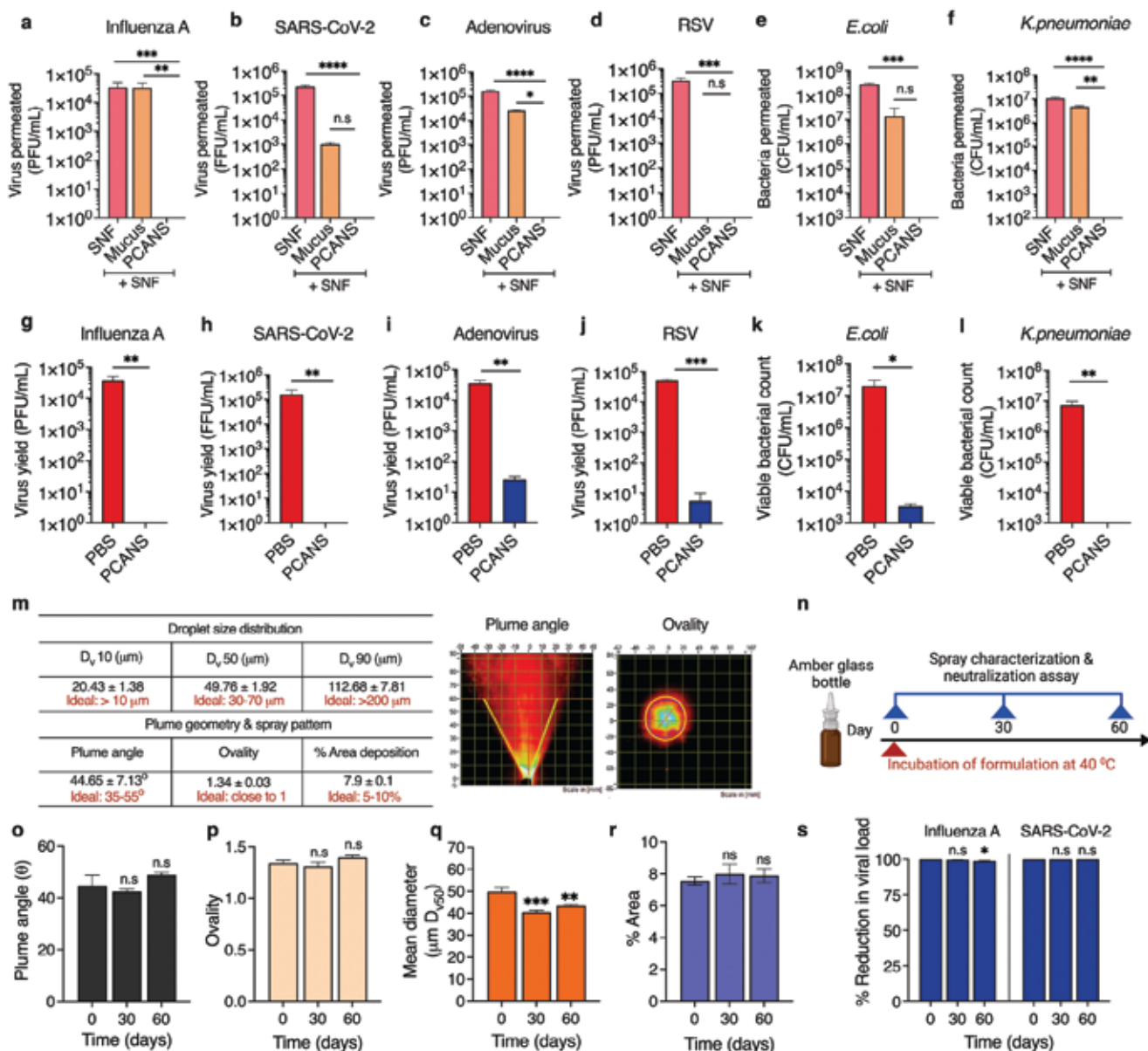


Figure 5. PCANS exhibits broad-spectrum physical barrier property with pathogen neutralization, and is sprayable and shelf-stable. a–d) Amount of different viruses that permeated within 4 h through a simulated nasal fluid (SNF)-coated cell strainer or a cell strainer coated with simulated mucus/SNF mixture or PCANS/SNF mixture. Virus permeation was quantified by plaque assay in MDCK cells (IAV), Vero E6 cells (SARS-CoV-2), and Hep-2 cells (RSV and adenovirus). **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. n.s., not significant. Amount of e) *E. coli* and f) *K. pneumoniae* that permeated within 4 h through an SNF-coated cell strainer or a cell strainer coated with simulated mucus/SNF mixture or PCANS/SNF mixture. Bacterial permeation was quantified using a CFU plate count method. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$. n.s., not significant. g–j) Viral titer for IAV, SARS-CoV-2, adenovirus, and RSV after treatment with PBS or PCANS. IAV and SARS-CoV-2 were incubated with PCANS for 10 min, while adenovirus and RSV were treated for 30 min. Anti-bacterial activity of PCANS against k) *E. coli* and l) *K. pneumoniae* using CFU plate count method after 30 and 10 min incubation, respectively. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. m) Spray characteristics of PCANS. The droplet size distribution of PCANS was analyzed using a laser diffraction system. Representative images of single time delay plume angle and ovality ratio, captured using a high-speed digital camera and laser light sheet. n) Experimental design to assess the stability of PCANS in accelerated temperature conditions (40 °C). PCANS was stored in glass amber bottles. Aliquots were taken at different time points to investigate spray characteristics and pathogen neutralization efficacy. o) Plume angle, p) ovality, q) mean droplet diameter and r) spray deposition area over a period of 60 days. *** $P < 0.001$, ** $P < 0.01$ compared to day 0, n.s., not significant. s) Percent reduction in the viral load of IAV and SARS-CoV-2 in their respective host cells after 10 min incubation with PCANS aliquoted at different time points in the stability study. * $P < 0.05$ compared to day 0, n.s., not significant. For a–f and o–s, P values were determined using one-way ANOVA with Tukey's post-hoc analysis. For g–l, P values were determined using a two-tailed t-test. Data are presented as Means $\pm$ SD of biological repeats ($n = 3$, each experiment performed at least twice).

Shelf-stability is a key attribute governing the translational potential of formulations. We tested shelf-stability of PCANS over 60 days at 40 °C temperature, as per the International Conference on Harmonization (ICH) guidelines for stability testing under accelerated storage conditions (Figure 5n). Over a period of 60 days, we observed no substantial variations in the spray characteristics, including plume angle, ovality, coverage area, and droplet size distribution (Figure 5o–r; Figure S12, Supporting Information). PCANS also displayed no changes in its neutralization activity over 60 days, resulting in >99.99% reduction in Influenza A and SARS-CoV-2 viral loads in the host cells upon 10 min of incubation (Figure 5s). Interestingly, in a separate stability study, we found that PEA's presence in PCANS is critical for minimizing bacterial growth after the formulation was challenged with *E. coli* (Figure S13, Supporting Information). This is consistent with previous reports,^[48] which have shown that PEA prevents bacterial growth in nasal sprays. Collectively, these data confirm the shelf-stability of PCANS.

2.6. PCANS Exhibits Prophylactic Activity In Vivo

Next, in a proof-of-concept study, we investigated the prophylactic efficacy of PCANS against respiratory infection in vivo. PR8, a mouse-adapted strain of H1N1 Influenza virus, was used to induce infection. PR8 is a highly virulent strain that induces severe respiratory infection in mice,^[60] and can be lethal at a dose of 10 PFU.^[61] In vitro assay revealed excellent potency of PCANS to neutralize 10⁶ PFU of PR8 within 10 min of incubation, resulting in >5-log fold (>99.99%) reduction of the viral load in host cells (Figure S14, Supporting Information). To demonstrate efficacy in vivo, PCANS or PBS (10 µL) was administered prophylactically to both the nostrils of healthy mice on day 0 (Figure 6a). Fifteen minutes later, animals were challenged intranasally with a free-flowing PR8 solution (10 µL/nostril), exposing mice to a total dose of 250 PFU, which has been previously used by other groups.^[62,63] Intranasal instillation of 10 µL of a fluorescent dye (DiR) solution into each nostril of mice showed a stronger fluorescent signal in the lungs compared to the nasal cavity (Figure S15, Supporting Information) at 2 h post-administration. This confirms that the liquid reached the lungs and was not primarily retained in the nasal cavity, suggesting that the viral suspension administered as a liquid bolus also reached the deep lungs.

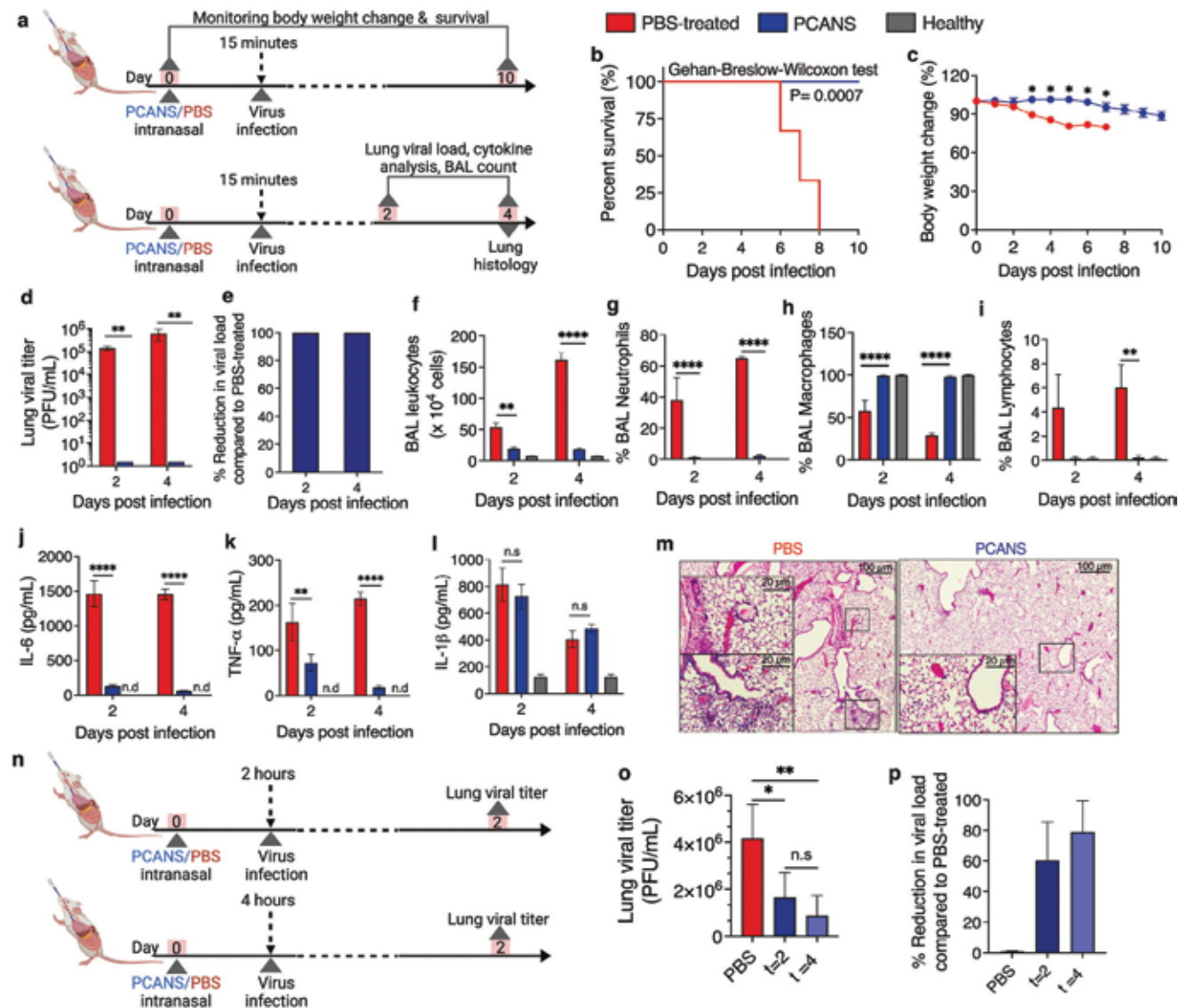
Remarkably, all mice in the PCANS-treated group survived for at least 10 days after the infection, whereas the PBS-treated group showed 100% lethality by day 8 (Figure 6b). Over 10 days, no discernible change was observed in the body weight of the PCANS-treated animals, while significant weight loss was observed for PBS-treated ones after 3 days post-infection (Figure 6c). PCANS also curtailed the lung viral titer to undetectable levels on days 2 and 4 post-infection, resulting in >5-log fold (>99.99%) reduction compared to PBS-treated mice (Figure 6d, e). Compared to healthy mice, mice infected with PR8 and treated with PBS showed significant differences in the levels of inflammatory cells, including leukocytes, neutrophils, lymphocytes, and macrophages in bronchoalveolar lavage (BAL) fluid (Figure 6f–i). Prophylactic treatment of mice with PCANS restored the levels of inflammatory cells in BAL fluid to normal. Additionally, cytokine profile from lung homogenate showed a significant reduction of

IL-6 and TNF-α levels in PCANS-treated mice, as compared to the PBS-treated group (Figure 6j–l). No reduction was, however, observed in the levels of IL-1β. Histological examination of lung sections revealed a substantial reduction in leukocyte infiltrates in PCANS-treated mice, as compared to the PBS-treated group, which showed an abundant presence of bronchial and alveolar infiltrates (Figure 6m). Overall, compared to PBS-treated mice, we observed a significant reduction in pulmonary inflammation score for PCANS-treated group (Figure S16, Supporting Information). An escalated dose challenge was performed to determine the potency of PCANS to neutralize a higher viral load of PR8 (500 PFU). Compared to the PBS-treated group, prophylactic treatment with PCANS significantly improved survival and body weight and reduced lung viral titer on days 2 and 4 post-infection (Figure S17, Supporting Information). PCANS also protected mice against the PR8 challenge performed after 2 and 4 h of prophylactic treatment, as evident from significant reduction observed in lung viral titer on day 2, as compared to the PBS-treated group (Figure 6n–p). Specifically, average reductions of 60% and 78% in lung viral titer were observed for 2 and 4 h challenge groups, respectively; however, there was no statistically significant difference between PCANS-mediated reductions observed in animals challenged after 2 versus 4 h. These data clearly indicate the potential of nasally administered PCANS to protect against respiratory infection in mice for at least 4 h.

3. Discussion

We report PCANS – a radically simple and multi-modal pre-exposure prophylactic nasal spray to offer protection against respiratory pathogens. Unlike vaccines, which are pathogen-specific and exhibit reduced efficacy as the pathogen mutates,^[64] PCANS has the potential to offer broad spectrum protection against a wide range of pathogens. In a proof-of-concept study performed in mice, a single intranasal dose of PCANS was effective against supra-lethal dosages of a highly virulent mouse-adapted strain of H1N1 Influenza virus (PR8), and efficiently reduced the lung viral titer providing protection for at least 4 hours. This underscores the potential utility of PCANS as an additional layer of protection in conjunction with vaccines to minimize pathogen load, considering that vaccines alone often achieve only partial reduction. For example, in a clinical study, participants vaccinated with BNT162b2 and mRNA-1273 had only 40 percent less detectable virus compared to those who were unvaccinated when infected.^[65]

To our knowledge, PCANS is the first multi-modal chemoprophylactic approach, equipped with three critical attributes that involve 1) capturing pathogen-laden respiratory droplets in the nasal cavity, 2) preventing pathogen transport via nasal lining and 3) neutralizing a broad spectrum of pathogens. This is the first report of a chemoprophylactic nasal spray that exhibits neutralization activity against a broad spectrum of pathogens, including both bacteria and viruses (enveloped and non-enveloped), and the first that has shown >4 log-fold reduction in pathogen load across multiple bacteria and viruses. Previously developed nasal sprays have demonstrated limited effectiveness with only a 1–2.5-log fold reduction in pathogen load. For instance, compared to xylitol-based formulations pHOXWELL and Xlear, which reduced viral titer of SARS-CoV-2 by 2 and 2.5 log fold,



respectively.^[23,66] PCANS demonstrated a remarkable 5 log fold reduction. In our study, iota + kappa carrageenan, the key component of a commercially available nasal spray- AGOVIRAX®, resulted in only 1-log fold reduction in the viral loads of both IAV and SARS-CoV-2 within 10 min, while PCANS showed >4 log fold reduction for both IAV and SARS-CoV-2 within 10 min. The superior prophylactic effect of PCANS was also demonstrated in vivo. Compared to a previous study,^[21] where multiple dosages of iota + kappa carrageenan showed 70% survival of mice in a PR8 model of infection, a single dose of our formulation showed 100% survival of mice. To our knowledge, this is the first chemoprophylactic strategy to show 100% protection within a pre-clinical model of respiratory virus infections. Additionally, we also demonstrated >99.99% reduction in lung viral titer. On the other hand, iota + kappa carrageenan only showed 75% reduction.^[21] PCANS also exhibits an unprecedented residence time of 8 h in the mouse nasal cavity and provides protection for at least 4 h. To our knowledge, this is the longest nasal residence time that has been reported for nasal sprays in mice. The interaction of PCANS with mucosal tissue and its prolonged retention time involve three key mechanisms: (1) mucoadhesive forces between the biopolymers (gellan and pectin) and the mucin proteins in the mucosal layer,^[67,68] (2) gelation of PCANS, facilitated by cross-linking of gellan in the presence of physiological electrolytes in nasal fluid, which intercalates with mucopolysaccharides to form a hydrogel mesh, preventing nasal dripping, and (3) the presence of tween-80, which has been previously shown to slow mucociliary clearance by reducing cilia beat frequency,^[69] thereby enhancing the nasal retention of PCANS. Such a long nasal residence time would potentially minimize dosage frequency in future clinical studies, offering an advantage over previously developed chemoprophylactic approaches, including SaNOTize, which require 3–6 doses per day.^[20,29] Finally, to our knowledge, this is the first nasal formulation that can effectively trap large respiratory droplets from the air we breathe in, which would be essential for maximizing its preventive effectiveness in future clinical studies.

The “drug-free” nature of PCANS is favorable for the regulatory process, which could be tedious for chemoprophylactic approaches based on investigational new drugs such as IgM-14.^[70] Also, since all the components used in PCANS are commercially available off-the-shelf and require simple mixing without chemical modifications, our approach is amenable to scale-up and large-scale manufacturing. PCANS is also safe for daily administration, as demonstrated in mice, which is a significant advantage over previously developed povidone iodine-based anti-viral nasal sprays,^[19,30] which are associated with iodine burns, thyroid toxicity, and disruption of the mucosal barrier, constraining repeated administration. Similarly, frequent use of a nitric oxide (NO)-inducing nasal spray (SaNOTize), which has shown potential in post-exposure prophylaxis of SARS-CoV-2 infection, can result in elevated Th2 cytokines, which mediate autoimmune disorders.^[71] In addition, excessive NO can cause tissue damage and cell death.^[29] The incidence of such adverse effects with PCANS is likely to be low, as the formulation is devoid of immunomodulatory molecules such as NO and steroids.

Our study has several strengths. First, the components constituting PCANS were identified via rigorous in vitro and in vivo screenings of excipients from the IID and GRAS list of the FDA,

and their different concentrations and combinations. These extensive screening experiments were aimed to optimize the key parameters, including sprayability, mucoadhesiveness, capture of respiratory droplets, physical barrier property, broad spectrum pathogen neutralization activity, and nasal residence time. Our comprehensive investigations allowed us to construct an unprecedented, multi-modal chemoprophylactic strategy. Second, to evaluate the respiratory droplet capturing ability of PCANS, we used a 3D- model of human nasal cavity (Koken cast), which has been previously used for in vitro evaluation of nasal drug delivery, as it replicates all the anatomical intricacies of human nasal cavity.^[56] We measured capture efficiency by comparing the fold increase in rhodamine B fluorescence intensity in the PCANS-coated nasal cavity compared to the one coated with mucus. This provided a robust and reproducible measurement. We also attempted to measure droplet capture efficiency by counting the droplets that passed through the model. To achieve this, we connected the nasal cavity cast to an aerosol monitoring system. However, the readings were inconsistent due to technical issues like ambient dust inflow through gaps in the nebulizer-nasal cavity connector and non-specific droplet deposition in the connector, leading to high variability. Third, physical barrier property of different biopolymers was evaluated by two complementary techniques – quantification of viral and bacterial transport using plaque-forming and colony forming assays as well as quantification of the transport of small molecule dye using fluorescence spectroscopy. Fourth, we demonstrated broad spectrum physical barrier property and neutralization ability of PCANS in five different pathogens – three enveloped viruses (IAV, SARS-CoV-2, RSV), one non-enveloped virus (adenovirus), and two bacteria (*E. coli* and *K. pneumoniae*). Lastly, to demonstrate prophylactic efficacy of PCANS in vivo, we used a highly virulent mouse-adapted strain of H1N1 Influenza virus (PR8) that induces severe respiratory infections in mice.^[60] Prophylactic efficacy of PCANS was demonstrated against three different dosages of the virus, which were 10–50 times higher than the previously established lethal dose for PR8 in mice.^[61] Due to its prolonged nasal residence time, PCANS was effective for at least 4 h after nasal administration.

Our study also has certain limitations, and there are additional questions that need to be answered. First, for the in vivo efficacy study, we instilled the viral suspension intranasally as a liquid bolus. Although aerosol administration of virus more closely recapitulates real-life exposure in humans, intranasal inoculation as a liquid bolus is a widely accepted standard in mouse models.^[72,73] Studies have shown that both methods result in comparable levels of morbidity, mortality, and viral titers in lung and nasal tissues for different influenza viruses as well as SARS-CoV-2.^[74,75] Thus, our choice of intranasal inoculation is scientifically justified. Excitingly, with intranasal inoculation—where the virus’s contact time with PCANS is limited to just a few seconds—we observed a greater than 99.99% reduction in lung viral titers and improved animal survival for 250 PFU dosage of PR8 virus. This underscores the significance of our findings. However, the 500 PFU group showed higher viral titer on day 4 and an early mortality compared to 250 PFU. We believe that reduced efficacy at 500 PFU might be partly, if not entirely, attributed to the limited contact time of virus particles with PCANS. Inhalation administration of the virus would deposit pathogen-laden

respiratory droplets on the PCANS layer, providing significant contact time between the pathogen and PCANS, potentially enhancing its prophylactic efficacy. Similar to the virus inoculation, we also instilled PCANS as a liquid bolus intranasally, while in humans PCANS would be sprayed using a metered dose spray pump, which is technically difficult to recapitulate in mice due to significant difference in their respiratory tract anatomy as compared to humans. To overcome these limitations, future studies should evaluate the efficacy of PCANS in large animal models, for example non-human primates (NHPs) that allow the administration of PCANS as a nasal spray and can be subsequently challenged with virus-laden respiratory droplets as an aerosol. Second, compared to >99.99% reduction in lung viral titer achieved by PCANS for PR8 challenge after 15 min, a 78% reduction was observed when the challenge was performed after 4 h. This can be largely explained by clearance of PCANS in the nasal cavity, resulting in significantly less formulation available to capture/neutralize the virus at 4 h as compared to 15 min. The residence time study with DiR-loaded PCANS showed ~50% clearance of PCANS in the first 4 h. This was also the reason for not evaluating the prophylactic efficacy of PCANS beyond 4 h in this study. Since mucociliary clearance in NHPs and humans is slower than mice,^[76] future efficacy studies in large animal models are warranted to accurately determine the local pharmacokinetics of PCANS and duration of protection following a single administration. Notably, the 78% reduction in lung viral titer, as observed in our study after 4 h of prophylactic treatment is still an improvement over 71% reduction observed with an inhalable formulation – spherical hydrogel inhalation for enhanced lung defense (SHIELD), developed recently as a prophylactic approach against SARS-CoV-2.^[77] Finally, although we demonstrated broad spectrum activity of PCANS against multiple pathogens in vitro, our proof-of-concept in vivo efficacy study only focused on PR8. Future studies should also evaluate the prophylactic efficacy of PCANS against other pathogens to confirm the broad-spectrum activity in vivo, and this should be ideally performed in NHPs to mimic the virus challenge and PCANS administration in humans.

4. Conclusion

PCANS presents a promising chemoprophylactic approach against respiratory infections. Besides its potential to act as a first line of defense against respiratory pathogens and emerging variants for which there are no vaccines available, our approach could also be potentially used as an added layer of protection with existing vaccines. Given its broad-spectrum prophylactic activity and shelf stability, we anticipate PCANS holds the potential for global distribution, especially in countries with low vaccination rates against respiratory pathogens. Alongside, the benefits of PCANS can also be extended to immunocompromised patients, high-risk individuals with co-morbidities, and vaccine-hesitant populations. Its pocket-sized spray format allows for easy portability, making it convenient to carry during social gatherings and travel. With these significant benefits, we believe PCANS will experience rapid widespread adoption, enhancing the accessibility of respiratory infection prevention. By enabling people to breathe clean and minimizing the transmission of respiratory infections,

PCANS could potentially play a pivotal role in safeguarding public health worldwide.

5. Experimental Section

Preparation of Biopolymer Solutions and PCANS: Biopolymer solutions were prepared by the addition of the biopolymer (0.2 to 2% w/v) to ultrapure deionized sterile water (Invitrogen). The solution was then mixed to attain a homogenous mixture with slight heating at 60 °C. Biopolymers including gellan (Gelzan), pectin, carboxymethylcellulose (CMC), hydroxypropyl methylcellulose (HPMC), carrageenan, xanthan gum, and Carbopol were purchased from Sigma Aldrich. To prepare PCANS, 0.4% w/v gellan and 1.5% w/v pectin solutions were mixed in a ratio of 1:1, followed by the addition of tween-80 (Sigma Aldrich) to obtain a final concentration of 0.05% w/v. The solution was then supplemented with benzalkonium chloride (BKC) (Sigma Aldrich) and subjected to immediate mixing by pipetting several times to yield 0.01% w/v in the solution. Finally, 0.25% w/v phenethyl alcohol (Sigma Aldrich) was added, and the pH of the solution was adjusted to 5.5. For cell culture experiments and in vivo efficacy study, the individual components of PCANS were sterile filtered using 0.2 µm PVDF syringe filters (EMD Millipore) and combined as described above.

Preparation of Simulated Nasal Fluid (SNF) and Simulated Mucus: SNF was prepared by dissolving 1.32 g sodium chloride (150 mM), 447 mg potassium chloride (39.9 mM), and 88.5 mg calcium chloride (5.3 mM) in 150 mL ultrapure deionized sterile water and filtered using 0.2 µm filter.^[78] The healthy simulated mucus was formulated by dissolving 0.6 mg mucin from porcine stomach Type II (Sigma Aldrich), 0.8 mg mucin from porcine stomach Type III (Sigma Aldrich), 0.32 mg bovine serum albumin (Sigma Aldrich) in 10 mL ultrapure deionized water containing 20 mM HEPES buffer and 38 mM sodium chloride solution.^[79] The mixture was stirred vigorously under slight heating to attain a homogenous solution.

Rheological Measurements: Dynamic viscosity behavior of biopolymer solutions was evaluated using a rotational rheometer (Discovery HR-2, TA Instruments) using a 40 mm diameter cone with a geometry angle of 1°. Samples were subjected to a linear shear rate ramp up to 40 s⁻¹ at 25 °C to mimic the strain encountered by the formulation when actuated through the nozzle of the spray device. The viscosity of the biopolymer solution was measured during the upward ramp in triplicates. The sol-gel transition of biopolymer solutions with and without the presence of SNF was evaluated by rotational rheology. The mechanical strength in terms of storage modulus was assessed by applying amplitude sweep with a varying oscillatory strain at 1 Hz at 37 °C.

Ex Vivo Mucosal Retention Study: Tissue harvested from sheep was cut open to expose the mucosal surface and trimmed down to 75×26 mm. Mucosal tissue was then mounted on a glass slide facing upwards and positioned at 45° to align it with the spray actuation angle. The tissue was initially moistened with SNF using a generic nasal spray device, and excessive fluid was removed with sterile wipes. Brilliant green dye (Sigma Aldrich) loaded polymeric solution was sprayed, keeping the spray nozzle tip at a distance of 5 cm from the slide surface. The slides were examined for runoff/drip after 4 h of spraying. The distance traveled by the polymer solution down the glass slide from the bottom end of formulation deposited on mucosal tissue was measured as drip length. Drip length of free dye was considered 100%.

Cell Culture: Madin-Darby canine kidney cells (ATCC®) were cultured in T-175 flasks (CELLTREAT) at 37 °C and 5% CO₂ in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Invitrogen). Hep2 cells and Vero E6 cells (ATCC®) were cultured in T-75 flasks at 37 °C and 5% CO₂ in EMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Human nasal epithelial cells (ATCC®) were cultured in T-175 flasks at 37 °C and 5% CO₂ in EMEM supplemented with 10% FBS and 1% penicillin-streptomycin.

Production of NanoLuc Luciferase Expressing Recombinant SARS-CoV-2: All replication-competent SARS-CoV-2 experiments were performed in a BSL-3 facility at the Boston University National Emerging Infectious Diseases Laboratories. A recombinant SARS-CoV-2 virus expressing a Neon-

Green fluorescent protein (rSARS-CoV-2 mNG) was generously provided by the Laboratory of Pei-Yong Shei.^[80] To propagate the virus, 1×10^7 Vero E6 cells were seeded in a T-175 flask one day prior to propagation. The next day, 10 μ L of rSARS-CoV-2 mNG virus stock was diluted in 10 mL of Opti-MEM, added to cells, and then incubated for 1 h at 37 °C. After incubation, 15 mL of DMEM containing 10% FBS and 1% penicillin/streptomycin was added to cells. The next morning, media was removed, cells were washed with 1X PBS and 25 mL of fresh DMEM containing 2% FBS was added. Virus was incubated for an additional 48 h. The supernatant was collected at 72 h, filtered through a 0.22 μ m filter, and stored at –80 °C. The viral stock was thawed and concentrated by ultracentrifugation (Beckman Coulter Optima L-100K; SW32 Ti rotor) on a 20% sucrose cushion (Sigma-Aldrich) at 25000 $\times$ g for 2 h at 4 °C. Media and sucrose were then discarded, pellets were dried for 5 min at room temperature, and viral pellets were resuspended in 100 μ L of cold 1X PBS at 4 °C overnight. The next day, concentrated virus was combined, aliquoted and stored at –80 °C.

In Vitro Physical Barrier Assay: A 70 μ m pore size mesh cell strainer was coated with 15 μ L of mucus, or a biopolymer solution, or PCANS. The formulation was spread evenly using a sterile stainless-steel spatula with a tapered end. To facilitate in situ gelation, 15 μ L of SNF was added, covering the entire surface of the strainer. The strainer was placed in a 6-well plate containing 0.9 mL of serum-free DMEM (for virus/bacteria penetration) or ultrapure deionized water (for rhodamine B isothiocyanate penetration) in each well, and 0.1 mL of diluted virus ($\approx 1 \times 10^5$ PFU mL^{–1})/bacteria (1×10^7 CFU mL^{–1}) stock or rhodamine B isothiocyanate (1 mg mL^{–1}) was added to the upper compartment of the strainer. After 4 h of incubation at 37 °C, medium or deionized water from the bottom reservoir was retrieved, and the viral titer permeated through the hydrogel layer was quantified using plaque assay for IAV performed in MDCK cells, crystal violet staining for RSV performed in Hep-2 cells, immunostaining for adenovirus performed in Vero E6 cells, focus forming assay for SARS-CoV-2 in Vero E6 cells, and colony forming unit (CFU) plate count method for bacteria, as described in the following sections. The permeation of dye through biopolymer solution/mucus was quantified by measuring the fluorescence intensity using a microplate reader.

In Vitro Neutralization Assay with Influenza A: Neutralization activity of different excipients and PCANS was evaluated by plaque assay. MDCK cells were seeded at a density of 2–3 million cells per well in a 6-well plate and then incubated at 37 °C to achieve ≈ 80 –90% confluency one day before infection. On the day of infection, 50 μ L of HKx31 Influenza A virus (H3N2, 5×10^4 – 1×10^5 PFU mL^{–1}) (BEI Resources) in infection media (serum-free DMEM containing 3 mg mL^{–1} TPCK-trypsin) was pre-treated with 50 μ L of PCANS, biopolymer solution, surfactant solution, alcohol solution or PBS. Samples were vortexed for 10 seconds and incubated at 37 °C for 10 or 60 min. After incubation, samples were centrifuged for 1 min at 1000 RPM, and the supernatant was subjected to a 10-fold serial dilution until eighth dilution using infection medium. MDCK cells were then exposed to pre-treated virus dilutions for 1 h. After infection, an overlay growth medium containing 2X DMEM with 2% agarose (50:50) was poured onto the top of the cell monolayer and incubated for 72 h. The overlay was removed, and cells were then fixed using 1 mL of 10% formalin and left for 1 h at room temperature, followed by the addition of 1% crystal violet for 5–15 min. Wells were washed with water and left to dry out and PFUs were counted to determine the viral titer.

In Vitro Neutralization Assay with SARS-CoV-2: The day prior to infection experiment, 8×10^4 Vero E6 cells/well were plated in a 24-well plate. To perform neutralization assay, 50 μ L of PCANS, biopolymer solution, surfactant solution, alcohol solution or PBS was mixed with 8×10^4 PFU of SARS-CoV-2 mNG in 50 μ L of infection media (OptiMEM (Gibco) containing 3 mg mL^{–1} TPCK-trypsin), vortexed and centrifuged briefly prior to incubation at 37 °C for 10 or 60 min. After incubation, samples were centrifuged for 1 min at 1000 RPM and serially diluted 10-fold until eighth dilution with infection medium. Of each dilution, 200 μ L was then plated into a 24-well plate and incubated for 1 h at 37 °C prior addition of 800 μ L of 1.2% Avicel (Dupont). Following a 24 h incubation period at 37 °C, Avicel was removed, cells were washed with 1X PBS and fixed for 3 h with 10% neutral buffered formalin. Focal forming units (FFU) per mL were de-

termined by counting NeonGreen expressing foci using an Evos M5000 fluorescent microscope (Thermo Scientific).

In Vitro Neutralization Assay with Adenovirus and Respiratory Syncytial Virus: The broad-spectrum neutralization potency of PCANS was evaluated against adenovirus type 5 (ADV-5, ATCC, VR-2554) and respiratory syncytial virus strain A2 (RSV-A2, ATCC, VR-1540) using plaque assay. Briefly, the day prior to the infection, 1×10^5 Vero E6 cells/well or 1.5×10^5 Hep-2 cells/well were plated in a 24-well plate for ADV-5 and RSV-A2, respectively. On the day of infection, 50 μ L of PCANS was mixed with 50 μ L of virus (1×10^6 PFU/mL of ADV-5 and 2×10^6 PFU/mL of RSV-A2) in the infection media and incubated at 37 °C for 30 min. The pre-treated mixture was 10-fold serially diluted in infection media after the incubation. Cells were washed with serum-free media before infection and 200 μ L of each dilution was transferred to the cells for a 1 h incubation prior to the addition of a 1 mL overlay medium containing methylcellulose. Following a 72 h incubation, the overlay layer was removed, and cells were fixed using 10% formalin with subsequent immunostaining for Vero E6 cells and crystal violet staining for Hep-2 cells. Plaques were counted using a plaque reader (Bioreader-600-Va).

In Vitro Neutralization Assay with Bacteria: The neutralization potency of components and PCANS was studied against gram-negative bacteria including *E. coli* and *K. pneumoniae*. An overnight culture of bacteria was prepared in 5 mL tryptic soy broth (TSB, Sigma Aldrich) media. On the day of the experiment, bacteria suspension was adjusted to obtain an OD_{600nm} = 0.2, which corresponds to 10^8 CFU/mL. A 50 μ L of bacterial suspension in TSB media was incubated with 50 μ L of PCANS, biopolymer solution, surfactant solution or alcohol solution at 37 °C for 10 or 60 min. After incubation, the sample/bacteria mixture was 10-fold serially diluted in 1X PBS, and 10 μ L of each dilution was plated onto pre-poured LB (Luria Broth, HiMedia Laboratories Pvt Ltd) agar plates followed by an incubation of 16–18 h at 37 °C, 5% CO₂. The plates were then counted for CFUs.

In Vitro Assay for Co-Neutralization of Bacteria and Virus: An overnight culture of *E. coli* was prepared in tryptic soy broth (TSB, Sigma Aldrich) media, and the bacterial suspension was adjusted to obtain a titer corresponding to 10^9 CFU/mL. A 10 μ L bacterial suspension was added to 1 mL of PCANS at 37 °C for 60 mins. Following the incubation, a 100 μ L PCANS/bacteria mixture underwent a 10-fold serial dilution in 1X PBS, and a CFU assay was performed. The remaining PCANS/bacteria mixture was syringe filtered using a 0.22 μ m filter to remove the *E. coli*, and neutralization of SARS-CoV-2 was performed as described above.

Molecular Docking Simulation for Rhodamine B Isothiocyanate: Molecular docking simulations were conducted using Biovia Discovery Studio 2021 Client and PyRx (version 0.8) software to investigate the binding affinities and interaction types between rhodamine B isothiocyanate and various biopolymers. Initially, the 3D structures of rhodamine B isothiocyanate (CID: 44 134 929), gellan gum (SID: 443 546 310), pectin (CID: 854), HPMC (CID: 57 503 849), CMC (CID: 24 749), xanthan gum (CID: 47 933), Carbopol (CID: 91 824 753), and κ -carrageenan (SID: 481 108 992) was obtained in.sdf format from the PubChem database. These structures were imported into PyRx, where the OpenBabel toolkit was employed for energy minimization and geometric confirmation. The molecules were then converted to.pdbqt format and categorized either as macromolecules (rhodamine B isothiocyanate) or as ligands (biopolymers). Docking analyses were carried out using AutoDock Vina within PyRx, employing a grid box method with dimensions of 25 $\times$ 25 $\times$ 25 Å. After the docking process, the binding free energies (expressed in kcal/mol) of the rhodamine B isothiocyanate-biopolymer complexes were evaluated. These docked complex files were subsequently imported into Biovia Discovery Studio to analyze various interaction types, including hydrogen bonds, carbon-hydrogen bonds, and hydrophobic interactions between the dye molecule and biopolymers.

In Silico Modeling of Viral Protein Interactions Neutralizing Agents: In-silico binding analysis was conducted using AutoDock Vina (<https://vina.scripps.edu/>). The receptor binding domains of influenza hemagglutinin (PDBID: 4WE4), SARS-CoV-2 spike receptor-binding domain (PDBID: 6M0J) and Envelope Protein Transmembrane Domain (PDBID: 7K3G) were sourced from the Protein Data Bank (PDB) available at RCSB (<https://www.rcsb.org/>). To prepare for docking simulations, molecular

files of the ligands were first generated using Marvin Sketch and then converted into PDB format using PyMOL for 3D structural visualization. The protein and ligand files were converted into AutoDock-readable formats (.pdbqt) to facilitate docking simulations. The simulations were performed with the receptor binding domains of the proteins to predict the most favorable binding conformations. AutoDock Vina and PyMOL were used to visualize and analyze these conformations. The binding energies associated with the predicted conformations were reported. Furthermore, to gain insights into the potential interactions between protein and ligand, LigPlot (<https://www.ebi.ac.uk/thornton-srv/software/LigPlus/>) software was utilized. This tool provided a detailed visual representation of the molecular interactions, highlighting the key residues involved in binding.

TEER Assay and In Vitro Cytotoxicity of tween-80: RPMI 2650 cells were seeded on the apical part of Transwell inserts (6.5 mm polyester membrane $\approx 0.4 \mu\text{m}$ pore size, Corning) at a density of 1.5×10^5 cells/cm² in 0.1 mL EMEM. The basolateral compartment of the insert was filled with 0.6 mL EMEM media supplemented with 10% FBS followed by incubation at 37 °C. On day 4, the medium was removed from the top of the inserts, and media volume in the bottom well was reduced to 200 μL . Every 2 days the medium was changed, and TEER was measured. An epithelial volt ohmmeter (World Precision Instrument) was used to measure the impedance. Until the monolayer formed with a constant impedance around 12 Ω , cells were grown with an air-liquid interface. On day 12, TEER was measured prior to the treatment of cells with surfactants. 200 μL of medium containing Triton X-100 (0.1% w/v) or tween-80 at different concentrations was added to the insert. Plate was incubated at 37 °C for 4 h. After incubation, wells were replenished with fresh medium, and TEER was measured after 4, 5, 12, and 24 h. The cytotoxic effect of tween-80 at different concentrations was also studied on RPMI 2650 cells. Briefly, 20000 cells/well were seeded in a 96-well plate and incubated at 37 °C overnight to achieve 70–80% confluency. Tween-80 (0.01, 0.05, and 0.5% w/v) solution in 0.2 mL EMEM media was added to the wells, followed by an incubation for 24 and 48 h. The metabolic activity of RPMI 2650 cells was measured using an XTT (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide) assay kit (ATCC®) according to the manufacturer's protocol.

Capture of Respiratory Droplets: The inner surface of a glass twin impinger's (Copley Scientific) oropharyngeal region (denoted by red arrows in Figure 4a) was coated with SNF followed by spraying the gellan (0.2% w/v) and pectin (0.75% w/v) mixture without or with different concentrations of tween-20, tween-80 or BKC using a VP3 nasal spray pump (Aptar). Droplets with mass median aerodynamic diameter $>5 \mu\text{m}$ and laden with rhodamine B-loaded liposomes (size $\approx 400 \text{ nm}$) were generated using a jet nebulizer. Nebulized droplets were administered into the impinger under vacuum at a flow rate of 15 L min^{-1} for 3 min. The gel was retrieved, and fluorescence intensity was quantified at an excitation and emission wavelength of 543 and 580 nm. Rhodamine B-loaded liposomes were synthesized using the thin-film hydration method.^[81,82] Briefly, the lipids, DSPE-PEG (2000) amine (Avanti Polar lipids), cholesterol (Sigma) and L- α -phosphatidylcholine, hydrogenated (Soy) (HPC, Avanti Polar lipids) were dissolved in chloroform to prepare a 10 mg mL^{-1} lipid stock solution in 1:1:3 molar ratio. A 2 mL of lipid stock solution was added to a round-bottom flask containing 0.8 mL of rhodamine B isothiocyanate from a 1 mg mL^{-1} stock. The organic solvent was then evaporated using a rotary evaporator for 5 min to form a thin lipid layer. The lipid film was then hydrated using 10 mL ultrapure water (Invitrogen) and silica glass beads were added to the flask to suspend the lipid in the solution with vigorous shaking using the rotary evaporator at 40 °C for 45 min. The hydrated lipid suspension was sonicated (Probe sonicator) at 30% amplitude for 1 min with a 2 sec pulse on and off condition. The size of liposomes was then analyzed using a Zeta Analyzer (Malvern).

To emulate the capture of pathogen-laden droplets in the human nasal cavity, a 3D transparent, silicone human nose model (Koken Co, Ltd) was used. The anterior region of the Koken model was deposited with SNF followed by the gellan (0.2% w/v) and pectin (0.75% w/v) mixture or PCANS with a single actuation using a nasal spray pump (Aptar). Koken model was connected to a vacuum pump at an air flow rate of 15 L min^{-1} and rhodamine B-loaded liposomes were then nebulized for 1 min. The

model was disassembled to retrieve the formulation and captured dye-loaded droplets after nebulization. The capture of droplets was measured by quantifying the fluorescence intensity at an excitation and emission wavelength of 543 and 580 nm.

Spray Characterization: Multi-dose nasal spray vials were filled with water or gellan solution or PCANS. The pump (140 μL) with an insertion depth of 1.8 cm (Aptar) was used to study the spray characteristics including plume geometry, spray plume, and droplet size distribution. Three replicate measurements were performed for each sample. Plume geometry and spray pattern were measured using a Spray-View measurement system (Proveris Scientific, Hudson, MA) at a distance of 30 mm from the nozzle orifice of the actuator. This acquisition system employs a high-speed digital camera and laser light sheet to capture images. Data were analyzed using an image processing software, Viota. Actuation parameters including velocity, acceleration and hold time, and settings for camera and laser were kept identical across all the samples. Plume geometry measures the angle of plume ejected from the nozzle orifice. Ovality and plume area were evaluated to quantify the spray pattern of the samples. Ovality was defined as the ratio of maximum to minimum cross-sectional diameter of the spray plume. A uniform circular plume with an ovality close to 1 can be considered an optimal condition for nasal sprays.^[83] Droplet size analysis of samples was inspected using a Malvern Spraytec laser diffraction system. The FDA recommends reporting the measurements of size distribution data at D(v,0.1), D(v,0.5), and D(v,0.9) thresholds which correspond to the size of 10%, 50%, and 90% droplets by volume distribution, respectively.^[58] It was suggested to have droplet population with D(v,0.1) $> 10 \mu\text{m}$, D(v,0.5) between 30–70 μm and D(v,0.9) $< 200 \mu\text{m}$. Droplet populations smaller than $10 \mu\text{m}$ have a propensity to induce a non-targeted deposition at the lungs, and droplets greater than $200 \mu\text{m}$ tend to drip/run off the nasal cavity.^[58]

Shelf-Stability Study: PCANS (15 mL) was filled in a sterile multi-dose nasal sprays (Aptar) capped with the actuator. The nasal spray vials were stored at an accelerated temperature condition (40° C). Aliquots were retrieved at different time points and evaluated for neutralization activity against IAV and SARS-CoV-2 using plaque forming and focus forming assays, respectively, as described above. Aliquots were collected from three different vials. Similarly, 5 mL aliquots were used to evaluate the spray features, including spray pattern, plume geometry, and droplet size distribution.

Mice: Animal experiments were conducted according to ethical guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Brigham and Women's Hospital. Experiments were conducted in 6–8 weeks-old C57BL/6 mice (Jackson Laboratories, USA). Mice were maintained under pathogen-free conditions and randomly assigned to various experiment groups, irrespective of gender. The group size of animals in experiments was decided based on the minimum number of animals required to attain a statistical significance of $P < 0.05$ among different test groups. For mouse model of influenza infection, experiments were conducted in Biosafety Level 2 according to ethical guidelines approved by the Institutional Animal Care and Use Committee (IACUC-A4752-01) of Brigham and Women's Hospital.

In Vivo Biodistribution and Nasal Retention: Nasal retention of the formulation was performed in mice. Briefly, C57BL/6 mice were administered with 10 μL per nostril of free DiR (Thermofisher) or PCANS mixed with DiR at a final concentration of $10 \mu\text{g mL}^{-1}$. Mice were euthanized at different time points and nasal cavity was harvested and imaged using IVIS (Bruker's In-Vivo Xtreme optical and x-ray in vivo imaging system) at an excitation and emission wavelength of 680/700 nm. Vital organs such as lung, liver, spleen, kidney, and heart, were also imaged at 2 and 24 h time points. To determine the mechanism of long residence time, animals were intranasally instilled with DiR-mixed gellan (0.02% w/v) and pectin (0.75% w/v) mixture without or with BKC and tween-80. After 8 h, animals were euthanized to harvest and image the nasal cavity using Perkin Elmer IVIS Lumina II and the total flux was expressed in ($\text{p/sec/m}^2/\text{sr}$).

In Vivo Prophylactic Activity of PCANS: Mice were intranasally instilled with 10 μL PCANS or PBS into each nostril under brief anesthesia using isoflurane. After 15 min, animals were challenged with 250 or 500 PFU of PR8 intranasally. One cohort of animals was followed for body weight

changes and survival for a period of 10 days. Animals were euthanized when the body weight was reduced to 20%. Animals from a second cohort were euthanized either on day 2 or 4 after infection to enumerate lung viral titer, inflammatory cell count in bronchoalveolar lavage (BAL) fluid, and inflammatory cytokine levels in lung homogenate. BAL fluid was isolated by gently instilling saline solution into bronchioles with a catheter inserted through the trachea. The total cells and immune cell types from the collected BAL fluid were quantified using Diff-quick kit as per manufacturer's protocol. For lung viral titer and cytokine profiling, left lung was homogenized and centrifuged at 2000 g for 10 min at 4 °C to collect the supernatant. The obtained supernatant was further used for downstream assays. Viral titer was enumerated using plaque assay with MDCK cells, as detailed above. Cytokine profiling was performed using respective ELISA kits of IL-6, TNF- α , and IL-1 β (BioLegend) according to the manufacturer's protocol. Histopathology of the right lung was determined using hematoxylin and eosin staining, and inflammation scoring was performed as reported previously.^[84] To evaluate the time-dependent protection of PCANS, animals were challenged with 100 PFU of PR-8 via intranasal route after 2 or 4 h of PCANS or PBS treatment and euthanized on day 2 post-infection to quantify lung viral titer using plaque assay.

Statistics: Statistical analysis and graphing were conducted using Graphpad Prism. A one-way ANOVA with Tukey's post hoc analysis was used to compare multiple groups. Two-way ANOVA with Tukey's multiple comparison tests was used to analyze the data with two variables. To evaluate the efficiency of PCANS, survival plots were generated using the Kaplan-Meier survival curve, and the statistical significance of the results was analyzed using the Gehan-Breslow-Wilcoxon test. *P* values for the body weight changes were determined using one-way ANOVA with Brown-Forsythe post hoc analysis. A *P*-value of less than 0.05 was considered statistically significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors acknowledge the use of BioRender for creating schematic illustrations - Fig. 1, Figs. 4a, 4f, 4j, Fig. 5n, and Figs. 6a, 6n. in the manuscript. The authors acknowledge funding support from Gillian Reny Stepping Strong Center for Trauma Innovation at the Brigham and Women's Hospital (to NJ and JMK), Department of Anesthesiology, Perioperative, and Pain Medicine at the Brigham and Women's Hospital (to NJ), Fulbright-Nehru Postdoctoral Fellowship (to JJ), and Boston University (to FD) and the Peter Paul Career Development Award (to FD). The metered dose spray pumps were generously gifted by Aptar Inc. The authors acknowledge Integrated BioTherapeutics (IBT) Bioservices for evaluating the neutralization activity of PCANS against RSV and adenovirus.

Conflict of Interest

J.J., H.M.B., Y.T., and J.M.K have one pending patent based on the PCANS formulation described in this manuscript. N.J. and J.M.K are paid consultants, scientific advisory board members, and hold equity in Akita Biosciences, a company that has licensed IP generated by N.J. and J.M.K. that may benefit financially if the IP was further validated. The interests of N.J. and J.M.K. were reviewed and overseen by their institution in accordance with its conflict of interest policies.

Author Contributions

J.J., H.M.B., and J.R.Q contributed equally to this work. J.J., H.M.B., J.R.Q., Y.T., J.M.K., N.J. performed conceptualization. J.J., H.M.B., J.R.Q., D.K.,

E.B., D.L., D.P. was performed data curation. J.J., H.M.B., J.R.Q., D.K., E.B., D.L., D.P. performed data analysis. J.M.K., N.J. performed funding acquisition. J.J., H.M.B., J.R.Q., Y.M., E.B., P.S., K.S., O.S., R.N., E.A., S.R., J.K. performed investigation. J.J., H.M.B., J.R.Q., D.K., S.K., X.L.L., J.M., J.G., J.N.L., A.Y., F.D. performed methodology. Y.T., J.M.K., N.J. performed project administration. F.D., Y.T., J.M.K., N.J. performed supervision. J.J., H.M.B., J.R.Q., J.M.K., N.J. performed validation. J.J., H.M.B., S.R., N.J., wrote manuscript – original draft: J.J., H.M.B., D.K., F.D., Y.T., J.M.K., N.J. edited the manuscript.

Data Availability Statement

The data that support that findings of this study are available from the corresponding author upon reasonable request.

Keywords

antibacterial, antiviral, broad spectrum protection, nasal prophylaxis, nasal spray, pathogen capture, respiratory infections, virus neutralization

Received: May 3, 2024

Revised: August 18, 2024

Published online:

- [1] T. Ferkol, D. Schraufnagel, *Ann. Am. Thorac. Soc.* **2014**, *11*, 404.
- [2] M. K. Hossain, M. Hassanzadeganroudsari, V. Apostolopoulos, *Expert Rev. Vaccines* **2021**, *20*, 635.
- [3] J. L. Excler, M. Saville, S. Berkley, J. H. Kim, *Nat. Med.* **2021**, *27*, 591.
- [4] E. V. Bailey, F. A. Wilson, *JAMA Netw. Open* **2022**, *5*, e226108.
- [5] O. J. Watson, G. Barnsley, J. Toor, A. B. Hogan, P. Winskill, A. C. Ghani, *Lancet Infect. Dis.* **2022**, *22*, 1293.
- [6] J. Joseph, *Immuno* **2022**, *2*, 571.
- [7] M. G. Thompson, J. L. Burgess, A. L. Naleway, H. Tyner, S. K. Yoon, J. Meece, L. E. W. Olsho, A. J. Caban-Martinez, A. L. Fowlkes, K. Lutrick, H. C. Groom, K. Dunnigan, M. J. Odean, K. Hegmann, E. Stefanski, L. J. Edwards, N. Schaefer-Solle, L. Grant, K. Ellingson, J. L. Kuntz, T. Zunie, M. S. Thiese, L. Ivacic, M. G. Wesley, J. Mayo Lamberte, X. Sun, M. E. Smith, A. L. Phillips, K. D. Groover, Y. M. Yoo, et al., *N. Engl. J. Med.* **2021**, *385*, 320.
- [8] C. C. Wang, K. A. Prather, J. Sznitman, J. L. Jimenez, S. S. Lakdawala, Z. Tufekci, L. C. Marr, *Science* **2021**, *373*, 1979.
- [9] J. H. Ahn, J. M. Kim, S. P. Hong, S. Y. Choi, M. J. Yang, Y. S. Ju, Y. T. Kim, H. M. Kim, T. Rahman, M. K. Chung, S. D. Hong, H. Bae, C. S. Lee, G. Y. Koh, *J. Clin. Invest.* **2021**, *131*.
- [10] W. Sungnak, N. Huang, C. Bécavin, M. Berg, R. Queen, M. Litvinukova, C. Talavera-López, H. Maatz, D. Reichart, F. Sampaziotis, K. B. Worlock, M. Yoshida, J. L. Barnes, N. E. Banovich, P. Barbry, A. Brazma, J. Collin, T. J. Desai, T. E. Duong, O. Eickelberg, C. Falk, M. Farzan, I. Glass, R. K. Gupta, M. Haniffa, P. Horvath, N. Hubner, D. Hung, N. Kaminski, M. Krasnow, et al., *Nat. Med.* **2020**, *26*, 681.
- [11] A. B. Patel, A. Verma, *JAMA – J. Am. Med. Assoc.* **2020**, *323*, 2386.
- [12] Y. J. Hou, K. Okuda, C. E. Edwards, D. R. Martinez, T. Asakura, K. H. Dinno, T. Kato, R. E. Lee, B. L. Yount, T. M. Mascenik, G. Chen, K. N. Olivier, A. Ghio, L. V. Tse, S. R. Leist, L. E. Gralinski, A. Schäfer, H. Dang, R. Gilmore, S. Nakano, L. Sun, M. L. Fulcher, A. Livraghi-Butrico, N. I. Nicely, M. Cameron, C. Cameron, D. J. Kelvin, A. de Silva, D. M. Margolis, A. Markmann, et al., *Cell* **2020**, *182*, 429.
- [13] B. Xia, X. Pan, R. H. Luo, X. Shen, S. Li, Y. Wang, X. Zuo, Y. Wu, Y. Guo, G. Xiao, Q. Li, X. Y. Long, X. Y. He, H. Y. Zheng, Y. Lu, W. Pang, Y. T. Zheng, J. Li, L. K. Zhang, Z. Gao, *Cell Discov.* **2023**, *9*, 2.

- [14] S. Baur, M. Rautenberg, M. Faulstich, T. Grau, Y. Severin, C. Unger, W. H. Hoffmann, T. Rudel, I. B. Autenrieth, C. Weidenmaier, *PLoS Pathog.* **2014**, *10*, 1004089.
- [15] J. N. Weiser, D. M. Ferreira, J. C. Paton, *Nat. Rev. Microbiol.* **2018**, *16*, 355.
- [16] N. Ivanova, Y. Sotirova, G. Gavrilov, K. Nikolova, V. Andonova, *Pharmaceutics* **2022**, *14*, 530.
- [17] S. Williamson, L. Dennison, K. Greenwell, J. Denison-Day, F. Mowbray, S. Richards-Hall, D. Smith, K. Bradbury, B. Ainsworth, P. Little, A. W. A. Geraghty, L. Yardley, *BMJ Open* **2022**, *12*, e059661.
- [18] M. Morokutti-Kurz, M. Fröba, P. Graf, M. Große, A. Grassauer, J. Auth, U. Schubert, E. Prieschl-Grassauer, *PLoS One* **2021**, *16*, e0237480.
- [19] S. Frank, S. M. Brown, J. A. Capriotti, J. B. Westover, J. S. Pelletier, B. Tessema, *JAMA Otolaryngol. Head Neck Surg.* **2020**, *146*, 1054.
- [20] M. Tandon, W. Wu, K. Moore, S. Winchester, Y. P. Tu, C. Miller, R. Kodgule, A. Pendse, S. Rangwala, S. Joshi, *Lancet Region. Health – Southeast Asia* **2022**, *3*, 100036.
- [21] A. Leibbrandt, C. Meier, M. König-Schuster, R. Weinmüller, D. Kalthoff, B. Pflugfelder, P. Graf, B. Frank-Gehrke, M. Beer, T. Fazekas, H. Unger, E. Prieschl-Grassauer, A. Grassauer, *PLoS One* **2010**, *5*, e14320.
- [22] Y. Lin, S. Yue, Y. Yang, S. Yang, Z. Pan, X. Yang, L. Gao, J. Zhou, Z. Li, L. Hu, J. Tang, Q. Wu, S. Lei, Q. Tian, Y. Wang, Y. Hao, L. Xu, Q. Huang, B. Zhu, Y. Chen, X. Chen, L. Ye, *Clin. Infect. Dis.* **2023**, *76*, e336.
- [23] D. Balmforth, J. A. Swales, L. Silpa, A. Dunton, K. E. Davies, S. G. Davies, A. Kamath, J. Gupta, S. Gupta, M. A. Masood, A. McKnight, D. Rees, A. J. Russell, M. Jaggi, R. Uppal, *Journal of Clinical Virology* **2022**, *155*, 105248.
- [24] J. Joseph, H. M. Baby, S. Zhao, X. Li, K. Cheung, K. Swain, E. Agus, S. Ranganathan, J. Gao, J. N. Luo, N. Joshi, *Exploration* **2022**, *2*, 20210038.
- [25] M. Fröba, M. Große, C. Setz, P. Rauch, J. Auth, L. Spanaus, J. Münch, N. Ruetalo, M. Schindler, M. Morokutti-Kurz, P. Graf, E. Prieschl-Grassauer, A. Grassauer, U. Schubert, *Int. J. Mol. Sci.* **2021**, *22*, 13202.
- [26] A. Gaffar, H. Thacore SUNY buffalo Seiyoun Yun, A. Chemine, M. Ferrari BioqualUSA, L. Wattay, M. L. Peterson, *Preprint, Research Square* **2022**.
- [27] C. C. Go, K. Pandav, M. A. Sanchez-Gonzalez, G. Ferrer, *Cureus* **2020**.
- [28] R. J. A. Moakes, S. P. Davies, Z. Stamataki, L. M. Grover, *Adv. Mater.* **2021**, *33*, 2008304.
- [29] F. Lisi, A. N. Zelikin, R. Chandrawati, *Adv. Sci.* **2021**, *8*, 2003895.
- [30] D. Zarabanda, N. Vukkadala, K. M. Phillips, Z. J. Qian, K. O. Mfuh, M. J. Hatter, I. T. Lee, V. K. Rao, P. H. Hwang, G. Domb, Z. M. Patel, B. A. Pinsky, J. V. Nayak, *Laryngoscope* **2022**, *132*, 2089.
- [31] J. Pennington, P. Pandey, H. Tat, J. Willson, B. Donovan, *Drug Dev. Ind. Pharm.* **2008**, *34*, 923.
- [32] G. M. Eccleston, M. Bakhshaei, N. E. Hudson, D. H. Richards, *Drug Dev. Ind. Pharm.* **2000**, *26*, 975.
- [33] S. Lungare, J. Bowen, R. Badhan, *J. Pharm. Sci.* **2016**, *105*, 1209.
- [34] D. M. Kirchmayer, B. Steinhoff, H. Warren, R. Clark, M. in het Panhuis, *Carbohydr. Res.* **2014**, *388*, 125.
- [35] D. Schutz, C. Conzelmann, G. Fois, R. Groß, T. Weil, L. Wettstein, S. Stenger, A. Zelikin, T. K. Hoffmann, M. Frick, J. A. Muller, J. Munch, *Am. J. Physiol. Lung Cell Mol. Physiol.* **2021**, *320*, L750.
- [36] M. P. Dirauf, P. C. Wagner, A. S. Brauer, *J. Supercrit. Fluids* **2022**, *191*, 105762.
- [37] F. Elwinger, P. Pourmand, I. Furó, *J. Phys. Chem. C* **2017**, *121*, 13757.
- [38] H. R. Moreira, L. P. da Silva, R. L. Reis, A. P. Marques, *Polymers (Basel)* **2020**, *12*, 329.
- [39] M. J. Penn, M. G. Hennessy, *Appl. Math. Model.* **2022**, *112*, 649.
- [40] T. E. Robinson, R. J. A. Moakes, L. M. Grover, *Front Med Technol* **2021**, *3*, 687681.
- [41] P. D. Rakowska, M. Tiddia, N. Faruqi, C. Bankier, Y. Pei, A. J. Pollard, J. Zhang, I. S. Gilmore, *Commun Mater* **2021**, *2*.
- [42] D. W. Woolley, *J. Exp. Med.* **1949**, *89*, 11.
- [43] Q. Lin, J. Y. C. Lim, K. Xue, P. Y. M. Yew, C. Owh, P. L. Chee, X. J. Loh, *VIEW* **2020**, *1*, e16.
- [44] Y. Kobayashi, Y. Suzuki, *PLoS One* **2012**, *7*, 40422.
- [45] P. H. Pawłowski, *AIMS Biophys.* **2021**, *8*, 111.
- [46] J. M. Figueroa, M. E. Lombardo, A. Dogliotti, L. P. Flynn, R. Giugliano, G. Simonelli, R. Valentini, A. Ramos, P. Romano, M. Marcote, A. Michelini, A. Salvado, E. Sykora, C. Kniz, M. Kobelinsky, D. M. Salzberg, D. Jerusalinsky, O. Uchitel, *Int. J. Gen. Med.* **2021**, *14*, 6277.
- [47] A. Frediansyah, *Clin. Epidemiol. Glob. Health* **2021**, *12*, 100826.
- [48] N. A. Hodges, S. P. Denyer, G. W. Hanlon, J. P. Reynolds, *J. Pharm. Pharmacol.* **1996**, *48*, 1237.
- [49] R. C. Rowe, P. J. Sheskey, W. G. Cook, A. P. Association, M. E. Fenton, *Handbook of Pharmaceutical Excipients*, Pharmaceutical Press, London **2012**.
- [50] M. Pérez-Rodríguez, M. L. Cabo, E. Balsa-Canto, M. R. García, *Int. J. Mol. Sci.* **2023**, *24*, 12132.
- [51] S. Rugonyi, S. C. Biswas, S. B. Hall, *Respir. Physiol. Neurobiol.* **2008**, *163*, 244.
- [52] G. W. Hallworth, D. G. Westmoreland, *J. Pharm. Pharmacol.* **1987**, *39*, 966.
- [53] A. Cidem, P. Bradbury, D. Traini, H. X. Ong, *Front. Bioeng. Biotechnol.* **2020**, *8*, 581995.
- [54] H. Y. Choi, Y. H. Lee, C. H. Lim, Y. S. Kim, I. S. Lee, J. M. Jo, H. Y. Lee, H. G. Cha, H. J. Woo, D. S. Seo, *Part. Fibre Toxicol.* **2020**, *17*, 5.
- [55] B. Marple, P. Roland, M. Benninger, *Otolaryngol. – Head Neck Surg.* **2004**, *130*, 131.
- [56] P. G. Djupesland, J. C. Messina, R. A. Mahmoud, in *Ther. Deliv*, Newlands Press Ltd, London **2020**, pp. 485–495.
- [57] S. Gizurarson, *Biol. Pharm. Bull.* **2015**, *38*, 497.
- [58] J. D. Ehrick, S. A. Shah, C. Shaw, V. S. Kulkarni, I. Coowanitwong, S. De, J. D. Suman, *Sterile Product Development* **2013**, *6*, 99.
- [59] V. Kulkarni, C. Shaw, *Formulat. Characteriz. Nasal Sprays* **2012**.
- [60] M. Fukushi, T. Ito, T. Oka, T. Kitazawa, T. Miyoshi-Akiyama, T. Kirikae, M. Yamashita, K. Kudo, *PLoS One* **2011**, *6*, 21207.
- [61] A. T. Harding, G. D. Haas, B. S. Chambers, N. S. Heaton, *PLoS Pathog.* **2019**, *15*, 1008098.
- [62] N. Gonzalez-Juarbe, A. N. Riegler, A. S. Jureka, R. P. Gilley, J. D. Brand, J. E. Trombley, N. R. Scott, M. P. Platt, P. H. Dube, C. M. Petit, K. S. Harrod, C. J. Orihuela, *Cell Rep.* **2020**, *32*, 108062.
- [63] D. Gowda, M. Ohno, S. G. Siddabasave, H. Chiba, M. Shingai, H. Kida, S. P. Hui, *Sci. Rep.* **2021**, *11*, 20161.
- [64] X. He, C. He, W. Hong, J. Yang, X. Wei, *Med. Res. Rev.* **2023**, *43*, 932.
- [65] D. Planas, N. Saunders, P. Maes, F. Guivel-Benhassine, C. Planchais, J. Buchrieser, W. H. Bolland, F. Porrot, I. Staropoli, F. Lemoine, H. Péré, D. Veyer, J. Puech, J. Rodary, G. Baele, S. Dellicour, J. Raymenants, S. Gorissen, C. Geenen, B. Vanmechelen, T. Wawina - Bokalanga, J. Martí-Carreras, L. Cuypers, A. Sève, L. Hocqueloux, T. Prazuck, F. A. Rey, E. Simon-Loriere, T. Bruel, H. Mouquet, et al., *Nature* **2022**, *602*, 671.
- [66] M. L. Cannon, J. B. Westover, R. Bleher, M. A. Sanchez-Gonzalez, G. Ferrer, *bioRxiv preprint* **2020**.
- [67] G. Tatykhanova, V. Aseyev, S. E. Kudaibergenov, *Reviews and Advances in Chemistry* **2020**, *10*, 140.
- [68] N. Thirawong, J. Nunthanid, S. Puttipatkhachorn, P. Sriamornsak, *Eur. J. Pharm. Biopharm.* **2007**, *67*, 132.
- [69] J. Jiao, L. Zhang, *Allergy Asthma Immunol. Res.* **2019**, *11*, 306.
- [70] Z. Ku, X. Xie, P. R. Hinton, X. Liu, X. Ye, A. E. Muruato, D. C. Ng, S. Biswas, J. Zou, Y. Liu, D. Pandya, V. D. Menachery, S. Rahman, Y. A. Cao, H. Deng, W. Xiong, K. B. Carlin, J. Liu, H. Su, E. J. Haanes, B. A. Keyt, N. Zhang, S. F. Carroll, P. Y. Shi, Z. An, *Nature* **2021**, *595*, 718.
- [71] C. Bogdan, *Nat. Immunol.* **2001**, *2*, 907.

- [72] J. L. Americo, C. A. Cotter, P. L. Earl, R. Liu, B. Moss, *Proc Natl Acad Sci U S A* **2022**, 119, e2202069119.
- [73] N. A. Ilyushina, A. M. Khalkov, J. P. Seiler, H. L. Forrest, N. V. Bovin, H. Marjuki, S. Barman, R. G. Webster, R. J. Webby, *J. Virol.* **2010**, 84, 8607.
- [74] J. A. Belser, K. M. Gustin, J. M. Katz, T. R. Maines, T. M. Tumpey, *Virology* **2015**, 481, 107.
- [75] V. Fumagalli, M. Ravà, D. Marotta, P. Di Lucia, C. Laura, E. Sala, M. Grillo, E. Bono, L. Giustini, C. Perucchini, M. Mainetti, A. Sessa, J. M. Garcia-Manteiga, L. Donnici, L. Manganaro, S. Delbue, V. Broccoli, R. De Francesco, P. D'Adamo, M. Kuka, L. G. Guidotti, M. Iannaccone, *Sci Immunol* **2022**, 7, eabl9929.
- [76] S. Gizurarson, *Acta Pharm. Nord.* **1990**, 2, 105.
- [77] X. Mei, J. Li, Z. Wang, D. Zhu, K. Huang, S. Hu, K. D. Popowski, K. Cheng, *Nat. Mater.* **2023**, 22, 903.
- [78] U. C. Galgatte, A. B. Kumbhar, P. D. Chaudhari, *Drug Deliv* **2014**, 21, 62.
- [79] R. Hamed, J. Fiegel, *J. Biomed. Mater. Res A* **2014**, 102, 1788.
- [80] X. Xie, A. E. Muruato, X. Zhang, K. G. Lokugamage, C. R. Fontes-Garfias, J. Zou, J. Liu, P. Ren, M. Balakrishnan, T. Cihlar, C. T. K. Tseng, S. Makino, V. D. Menachery, J. P. Billello, P. Y. Shi, *Nat. Commun.* **2020**, 11.
- [81] M. J. Barea, M. J. Jenkins, M. H. Gaber, R. H. Bridson, *Int. J. Pharm.* **2010**, 402, 89.
- [82] H. Zhang, *Methods Mol. Biol.* **2017**, 1522, 17.
- [83] D. J. Farina, in *Handbook of Non-Invasive Drug Delivery Systems*, Elsevier, Amsterdam **2010**, pp. 247–290.
- [84] S. Awasthi, N. Rahman, B. Rui, G. Kumar, V. Awasthi, M. Breshears, S. Kosanke, *BMC Pulm Med* **2020**, 20.