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Potent Antiviral and Antimicrobial Polymers as Safe and Effective Disinfectants for the Prevention of Infections

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Disinfection using effective antimicrobials is essential in preventing the spread of infectious diseases. This COVID-19 pandemic has brought the need for effective disinfectants to greater attention due to the fast transmission of SARS-CoV-2. Current active ingredients in disinfectants are small molecules that microorganisms can develop resistance against after repeated long-term use and may penetrate the skin, causing harmful side-effects. To this end, a series of membrane-disrupting polyionenes that contain quaternary ammoniums and varying hydrophobic components is synthesized. They are effective against bacteria and fungi. They are also fast acting against clinically isolated drug resistant strains of bacteria. Formulating them with thickeners and nonionic surfactants do not affect their killing efficiency. These polyionenes are also effective in preventing infections caused by nonenveloped and enveloped viruses. Their effectiveness against mouse coronavirus (i.e., mouse hepatitis virus-MHV) depends on their hydrophobicity. The polyionenes with optimal compositions inactivates MHV completely in 30 s. More importantly, the polyionenes are effective in inhibiting SARS-CoV-2 by >99.999% within 30 s. While they are effective against the microorganisms, they do not cause damage to the skin and have a high oral lethal dose. Overall, these polyionenes are promising active ingredients for disinfection and prevention of viral and microbial infections.

1. Introduction

The Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2) crippled the world in 2020 as the virus spread quickly from Asia, across Europe, and into North and South America.[1] The number of infected people and deaths escalated quickly, which led to the declaration of a COVID-19 (Coronavirus disease 2019) pandemic on March 11, 2020 by the World Health Organization. SARS-CoV-2 is part of the *Coronaviridae* family, which are enveloped, positive-sense, single-stranded RNA viruses. Compared to the other coronaviruses, such as SARS-CoV and MERS-CoV, this virus has a larger threat due to its higher infectivity rates, longer incubation times, and delayed symptoms.[2] This virus transmits through respiratory droplets when an infected person sneezes, coughs, or speaks to a group of people at close proximity.[3] The virus enters another person when the person breathes in the droplets, or by touching a contaminated surface followed by rubbing their eyes, nose, or mouth. Therefore, in addition to wearing a facemask and avoiding touching our faces, other important hygiene practices are to wash our hands often with proper handwashing techniques and disinfect frequently touched surfaces.

Surface sanitizers for hands or hard surfaces are often a formulation of at least 60% alcohol and may contain other active ingredients, such as quaternary ammoniums.[4] Alcohols disrupt the lipid membranes and cause the rapid release of microorganisms' intracellular components.[5] The effectiveness of alcohols, primarily ethanol, and isopropanol, is concentration dependent and is optimal between 60% and 70% by solution in water, with a contact time of at least 1 min.[4b] However, bacteria are able to develop tolerance toward these short-chain alcohols.[6] A combination of active ingredients increases the efficacy of the hand sanitizers and reduce the contact time needed. Quaternary ammonium chloride (QAC) compounds such as benzalkonium chloride and chlorhexidine are cationic surfactants, commonly added to these formulations. QACs are generally lipophilic and solvate lipid membranes. An advantage of QACs is their relatively high tolerance toward the presence of contaminating organic matter.

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However, most QACs have molecular weight of less than 500, which allow them to penetrate the skin barrier.[7] Prolonged and repeated use could predispose individuals to sensitization and potentially induce allergic contact dermatitis. Furthermore, the increased exposure of QACs to the microbial community could also lead to increased antimicrobial resistance in bacteria.[8]

To this end, antimicrobial polymers have demonstrated to be able to address the urgent antibiotic resistance problem. Due to the increased number of cationic centers per molecule, polymeric quaternary ammoniums are effective antimicrobials.[9] Physicochemical properties and structural factors, such as average molecular weight,[10] polymer architecture (homopolymers, random, or block copolymers, branched polymers),[11] the position of hydrophobic groups relative to the cationic groups and amphiphilicity balance[12] have been found to affect their biocidal activity toward bacterial and fungal cells relative to their toxicity to red blood cells. Recently, we reported polyionenes that were synthesized by copolymerization of commercially available tetramethyl-1,3-diaminopropane and bis-halide comonomer containing rigid amide bonds, and these polymers had good antimicrobial activity.[13] Importantly, bacteria did not gain resistance to these polymers after repeated use. Though their use as disinfectants against bacteria and fungi is promising, they are not potent against viruses.

In this study, we synthesized various polyionenes from more hydrophobic monomers to enhance antiviral activity. The polyionenes were evaluated against a broad spectrum of bacteria, fungi, model nonenveloped virus (bacteriophage Salmonella P22) and model enveloped virus (mouse coronavirus that has strong biological resemblance to SARS-CoV-2) to determine their antimicrobial and antiviral activities. Their ability to kill microbes and virus within 30 s was also studied. The polyionenes were formulated with thickeners and nonionic surfactants to form hand sanitizers, which were investigated for antimicrobial activity. Moreover, the toxicity of the polyionenes was studied by measuring their oral median lethal dose and their skin compatibility after multiple topical uses on mouse skin in vivo.

2. Results

2.1. Synthesis and Characterization of the Polyionenes

A series of cationic polyionenes (**A**–**E**) was synthesized using commercially available monomers tetramethyl-1,3-diaminopropane **a**) and *α*,*α*'-dichloro-p-xylene **b**), and a previously reported monomer *N*,*N*'-(ethane-1,2-diyl)bis(4-(chloromethyl)benzamide) **c**).[13b,c] (**Scheme 1 (I)**). The amount of monomer **c** incorporated increased from polyionene A– E.

Similarly, polyionenes **X1** and **X2** were synthesized by replacing monomer **a** with the more hydrophobic monomer *N*,*N*,*N*",*N*"-tetramethyl-1,6-diaminohexane **f**), and replace monomer **c** with the more hydrophobic monomer **d** or **e** (Scheme 1 **(II)**). Polyionenes **Y** and **Z1** were synthesized using **f**, and the more hydrophobic monomer **g** or **h** as compared to the monomer **c**, while **Z2** and **Z3** (Scheme 1 **(III)**) were synthesized from additional commercially available monomers **b** (represented as R1 in Scheme 1 **(III)**) and 4,4'-bis(chloromethyl)biphenyl (**R2**), respectively. These polyionenes are more hydrophobic than the polymers **A**–**E**.

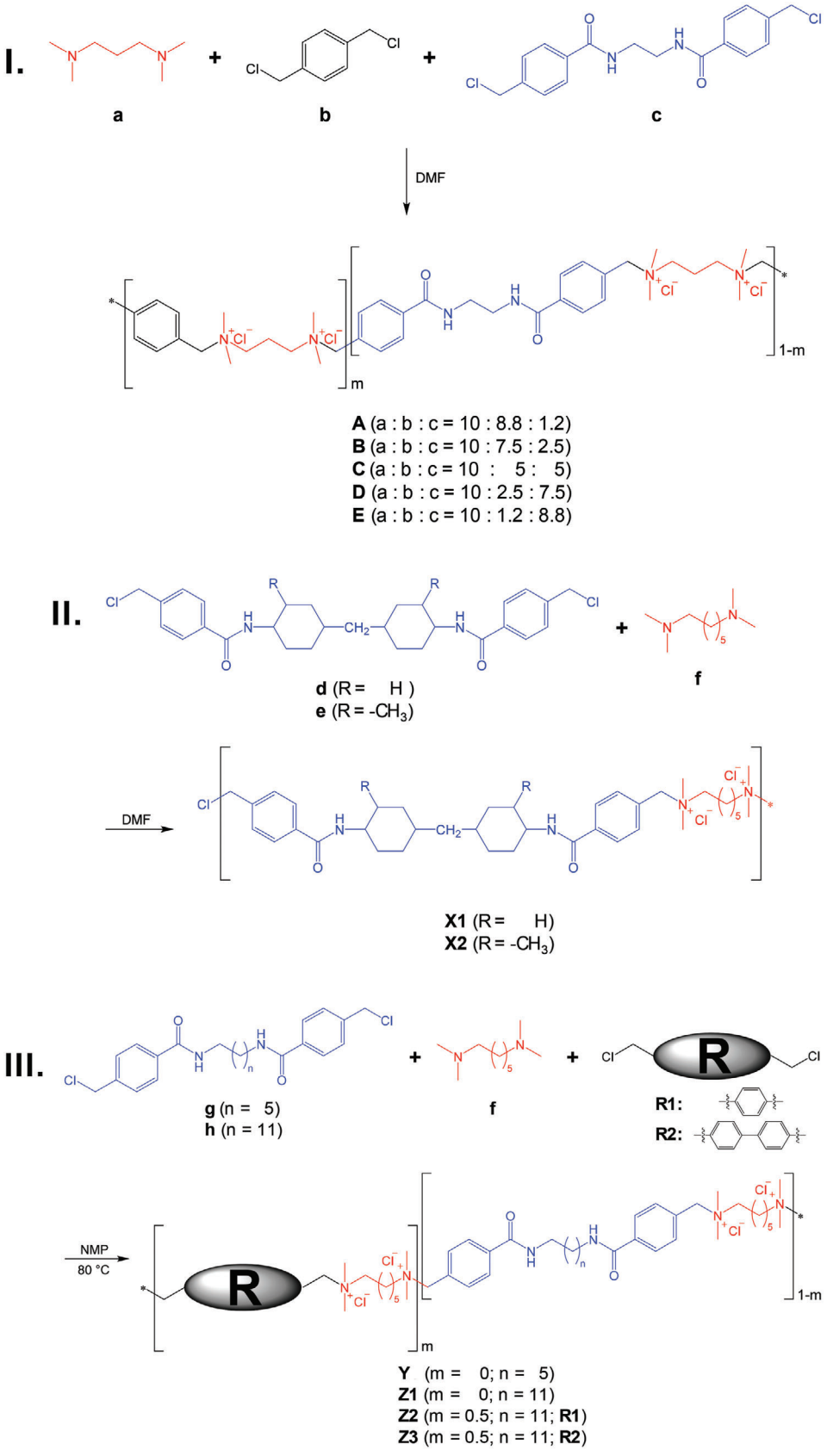
For all the polyionenes, the gel permeation chromatography (GPC) elution spectra showed a single unimodal peak (data not shown) and their number-average molecular weight (Mn) ranged from 5770 to 10 870, with the polydispersity indices (PDI) varying within 1.20–1.62. The compositions of the polymers were estimated from ^{1}H NMR measurements. **Figure 1** shows the proton spectrum of the polymer **Z3**. By quantitative comparisons of the integral intensities between the peak derived from the ethylene groups from monomer **f** at 2.00 ppm (H_d) with that of the ethylene protons from monomer **h** at 1.34 ppm (H_g), the molar ratio of the feed **f** and **h** in the polymer was determined to be 0.5:1, i.e., m is 0.5. In this way, all the compositions of the polymers were determined and summarized in Scheme 1.

2.2. In Vitro Broad Spectrum Antibacterial and Antifungal Activities

The susceptibility of 4 clinically relevant microbes toward the polyionenes was evaluated (**Table 1**). Polyionenes **A**–**E** showed broad-spectrum activities with MIC values ranging from 1.95 to 15.6 μg mL^{-1}, which are similar to commercially used active ingredients polyhexamethylene biguanide (PHMB) and chlorhexidine gluconate (Table S1, Supporting Information). The introduction of an increased amount of more hydrophobic monomer **c**) led to higher MIC values (from **A** to **E**) against *S. aureus*. There was no significant difference in MIC among **A**–**E** against other types of bacteria tested. Polyionenes **X1**–**Z3**, with the exception of Z2, had higher MICs ranging from 15.6 to 62.5 μg mL^{-1} as compared to polyionenes **A**–**E**. Based on the reduction in colonies observed from the agar plate assay, the polyionenes are bactericidal and fungicidal to the bacteria and fungi, respectively, at concentrations ranging from 2 to 4 times their respective MIC (Table 1). Polyionenes **A**–**E** showed negligible hemolysis at low concentrations with HC_{50} above 2000 μg mL^{-1} despite increasing the number of more hydrophobic monomer (Figure S1A, Supporting Information). As expected, the more hydrophobic polyionenes **X1**–**Z3** showed stronger hemolytic activity with HC_{50} values ranging from 16 μg mL^{-1} for **Z1** and 2000 μg mL^{-1} for **Y** (Figure S1B, Supporting Information).

To determine if the polyionenes were effective against drug-resistant bacteria, polyionenes **A** and **C** were tested against two patient-derived multidrug-resistant (MDR) strains of bacteria, methicillin-resistant *S. aureus* (MRSA), and New Delhi metallo-*β*-lactamase (NDM)-producing *K. pneumonia*e (**Table 2**). Similar to the drug-susceptible strains, the polyionenes have MICs and MBCs of 3.9–7.8 and 7.8–15.6 μg mL^{-1}, respectively. They reduced the number of bacteria by 5-log within 30 s at a concentration of 0.5 w/v%, demonstrating high potency against the MDR bacteria.

The change in the permeability of bacterial membrane was investigated after the treatments with polyionene by observing the binding of propidium iodide (PI, a fluorescent dye) to bacterial DNA (**Figure 2**). PI is a positively charged molecule and does not diffuse across intact membranes. *S. aureus* and *E. coli* were treated with either polyionene A or Z3 at concentrations equivalent to four times their respective MIC for 2 h. For both *S. aureus* and *E. coli*, the polyionene-treated groups had a much higher red fluorescence intensity as compared to the untreated control



Scheme 1. Synthesis and chemical structures of functional polyionenes A–E) (shown in **I**), **X1** and **X2** (shown in **II**), and **Y**, **Z1**, **Z2**, and **Z3** (shown in **III**).

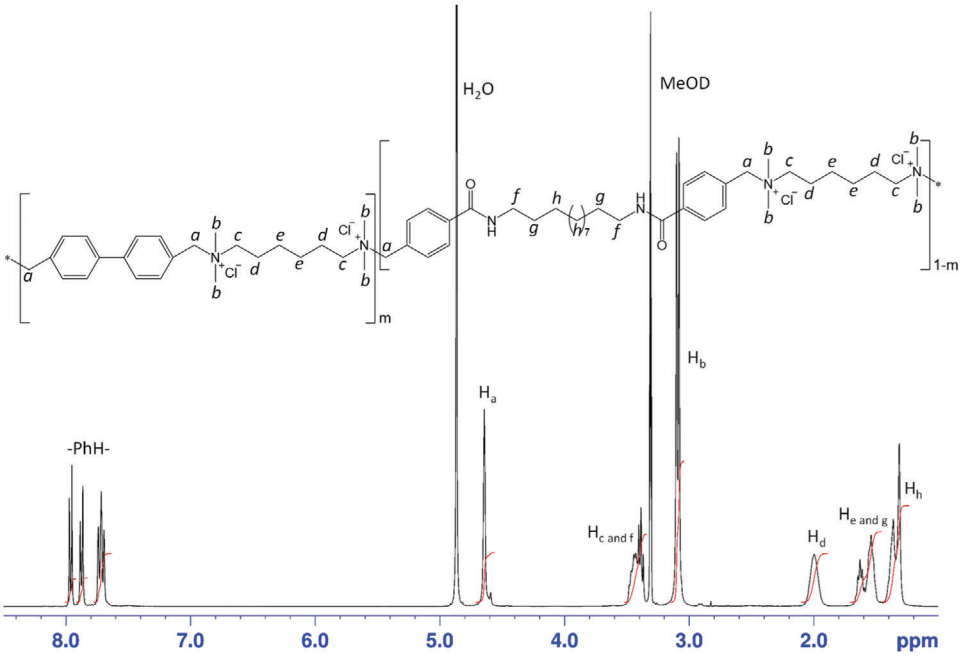


Figure 1. ^{1}H NMR spectrum of polyionene **Z3** in CD_3OD.

Table 1. Molecular weight (Mn), molecular weight distribution, minimum inhibitory concentrations (MIC, μg mL^{-1}), minimum bactericidal or fungicidal concentrations (MBC, MFC, μg mL^{-1}) of polyionenes and the polyionene concentration that causes 50% hemolysis (HC_{50}, μg mL^{-1}).

		S. aureus		E. coli		P. aeruginosa		C. albicians		
Polymer	Mn [g mol^{-1}]/PDI	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	HC_{50}
A	7930/1.34	1.95	7.8	3.9	15.6	3.9	15.6	7.8	15.6	>2000
B	7450/1.38	1.95	3.9	3.9	15.6	3.9	15.6	3.9	7.8	>2000
C	6750/1.41	7.8	15.6	7.8	15.6	7.8	31.3	1.95	3.9	>2000
D	6180/1.50	7.8	31.3	7.8	31.3	7.8	15.6	1.95	3.9	>2000
E	6550/1.46	7.8	31.3	7.8	31.3	7.8	15.6	3.9	7.8	>2000
X1[a)]	5770/1.62	15.6	31.3	15.6	15.6	31.3	62.5	31.3	62.5	1000
X2[a)]	5800/1.57	15.6	31.3	62.5	125	62.5	250	62.5	250	600
Y	10870/1.16	15.6	15.6	15.6	15.6	15.6	31.3	62.5	125	2000
Z1[a)]	5990/1.45	15.6	15.6	31.3	62.5	31.3	62.5	62.5	125	16
Z2[a)]	8760/1.30	7.8	7.8	7.8	15.6	7.8	15.6	15.6	15.6	80
Z3[a)]	10400/1.20	15.6	15.6	15.6	31.3	15.6	31.3	31.3	31.3	32

[a)] There was visible precipitation of media contents upon adding polymer solution to the broth.

group. This indicates that the bacterial membrane was disrupted by polyionene A or Z3, allowing PI to diffuse across the bacterial membrane and bind with the intracellular DNA.

2.3. Antibacterial and Antifungal Hand Wash and Hand Sanitizer Formulations

A hand wash formulation usually consists of 3 parts: a thickener, an antimicrobial agent, and a surfactant, whereas a rinse-free hand sanitizer is made without using a surfactant but with alcohol. (Hydroxypropyl)methylcellulose (HPMC) was used as a thickening agent, and octylphenoxy polyethoxyethanol, *N*-decyl-*b*-*D*-glucopyranoside and *N,N*-bis(2-hydroxyethyl)dodecanamide were employed as nonionic surfactants. Various hand wash (Formulation **5**, **6**) and sanitizer (Formulation **2–4**) formulations were prepared for evaluation of antimicrobial and antiviral activities in comparison with the commonly used small molecular antimicrobial agent chlorhexidine and polymer-based antimicrobial agent PHMB (**Table 3**).

Without the use of polyionenes, Formulation **1** did not have any antibacterial and antifungal properties (**Table 4**). However, when polyionene A was mixed in at the concentration of 0.5 w/v% to form hand sanitizer formulation **2** (without alcohol), **3** (containing 30 v/v% alcohol), and **4** (containing 50 v/v% alcohol), the

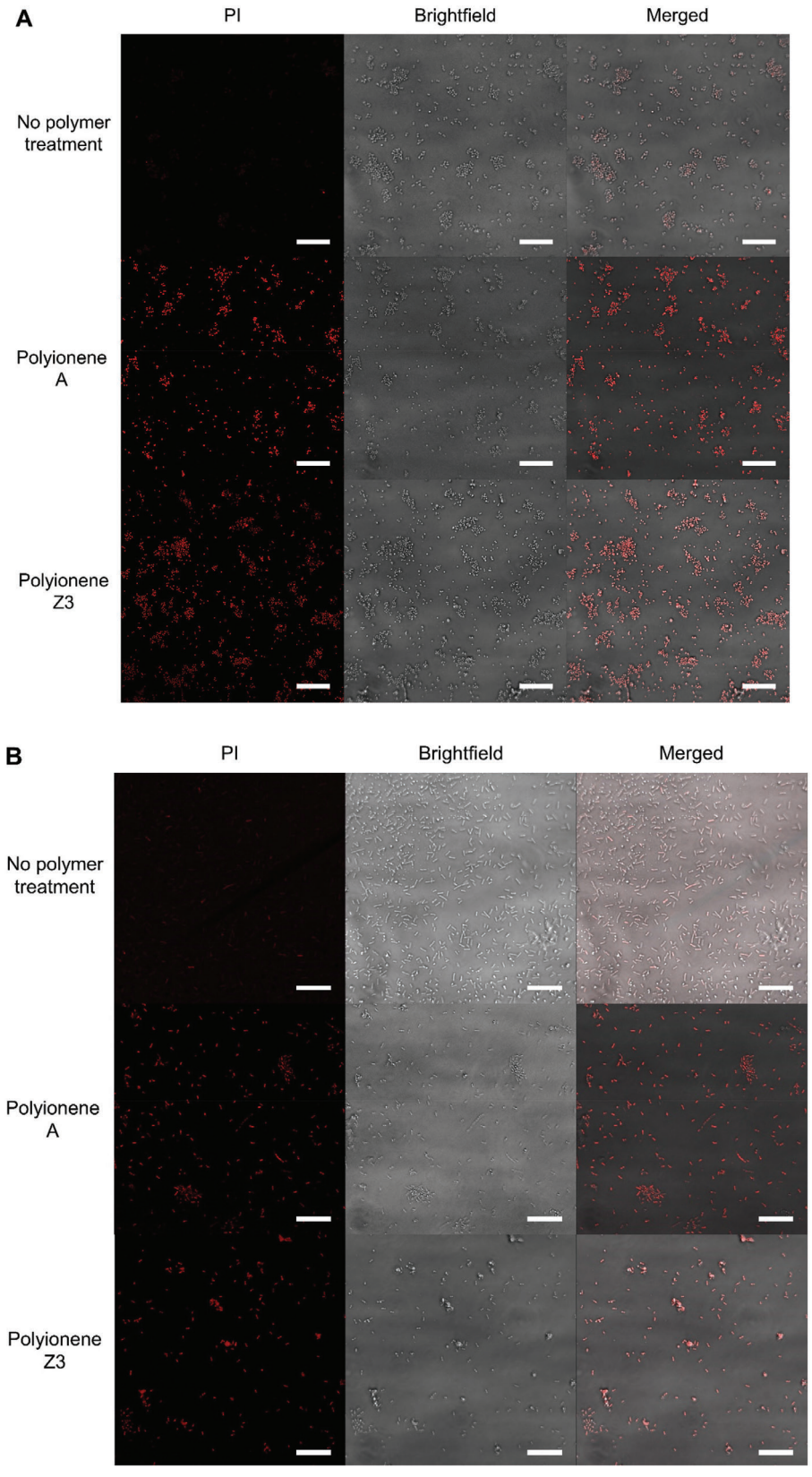


Figure 2. Confocal microscopic images of *S. aureus* A) and *E. coli* B) after 2 h treatment with polyionene A or Z3 at 4×MIC (For *S. aureus*: polyionene A: 8 μg mL^{-1}, Z3: 62.5 μg mL^{-1}. For *E. coli*: polyionene A: 16 μg mL^{-1}, Z3: 62.5 μg mL^{-1}). Red fluorescence indicates the location of propidium iodide. Scale bar represents 20 μm.

Table 2. MIC and MBC of polyionenes A and C against methicillin-resistant *S. aureus* (MRSA) and New Delhi metallo-β-lactamase (NDM)-producing *K. pneumonia*e.

	MRSA			NDM-producing K. pneumoniae		
Polymer	MIC	MBC	Killing efficiency[a)]	MIC	MBC	Killing efficiency[a)]
A	3.9	7.8	>LOD	7.8	7.8	>LOD
C	7.8	15.6	>LOD	7.8	7.8	>LOD

[a)] The polymer concentration tested is 0.5 w/v% and the contact time is 30 s. The average limit of detection is 99.94%.

Table 3. Compositions of the hand wash and hand sanitizer formulations.

	Composition
Formulation 1	1 w/v% hydroxypropyl methyl cellulose (HPMC)
Formulation 2	1 w/v% HPMC + 0.5 w/v% polyionene A or polyionene Z3
Formulation 3	1 w/v% HPMC + 0.5 w/v% polyionene A or polyionene Z3 + 30 w/v% pure alcohol
Formulation 4	1 w/v% HPMC + 0.5 w/v% polyionene A + 50 w/v% pure alcohol
Formulation 5	1 w/v% HPMC, 5 w/v% octylphenoxy polyethoxyethanol, 0.5 w/v% polyionene A, 5 w/v% decyl β-D-glucopyranoside
Formulation 6	1 w/v% HPMC, 5 w/v% octylphenoxy polyethoxyethanol, 0.5 w/v% polyionene A, 5 w/v% *N*,*N*-bis(2-hydroxyethyl)dodecanamide
Formulation 7	4 w/v% chlorhexidine
Formulation 8	0.004 w/v% polyhexamethylene biguanide

killing efficiencies against bacteria and *C. albicans* were above the limit of detection (LOD, >99.9%) within 30 s. There were no colonies on the agar plates, indicating that the polyionenes had high antimicrobial efficacy. Comparing Formulation **2** (without ethanol) with Formulations **3** and **4** (with ethanol), the ethanol did not influence the antimicrobial efficiency, but decreased the drying time to less than 1 min. The presence of the nonionic surfactants did not affect the antimicrobial efficacy of the polyionene in hand wash formulations **5** and **6** (**Table 5**). The killing efficiency of Formulations **5** and **6** was greater than 99.99% within 30 s, and no colonies were found on the agar plates. A similar observation was observed when polyionene A was replaced with polyionene Z3 (Table S2, Supporting Information). Fast killing efficiency was also observed for chlorhexidine (Formulation **7**) and

Table 4. Antimicrobial activity of hand sanitizers with ethanol after 30 s contact time.

	Antimicrobial efficiency [%]			
	S. aureus	E. coli	P. aeruginosa	C. albicans
Limit of detection (LOD, %)	99.995	99.991	99.952	99.943
Formulation 1	*N.S.*[a)]	*N.S.*	*N.S.*	*N.S.*
Formulation 2	>LOD	>LOD	>LOD	>LOD
Formulation 3	>LOD	>LOD	>LOD	>LOD
Formulation 4	>LOD	>LOD	>LOD	>LOD

[a)] *N.S.*: There is no significant difference when compared with untreated MHB group.

Table 5. Antimicrobial activity of hand sanitizers without ethanol after 30 s contact time.

	Antimicrobial efficiency [%]			
	S. aureus	E. coli	P. aeruginosa	C. albicans
Limit of detection (LOD, %)	99.994	99.997	99.997	99.990
Formulation 5	>LOD	>LOD	>LOD	>LOD
Formulation 6	>LOD	>LOD	>LOD	>LOD
Formulation 7	>LOD	>LOD	>LOD	>LOD
Formulation 8	>LOD	>LOD	>LOD	>LOD

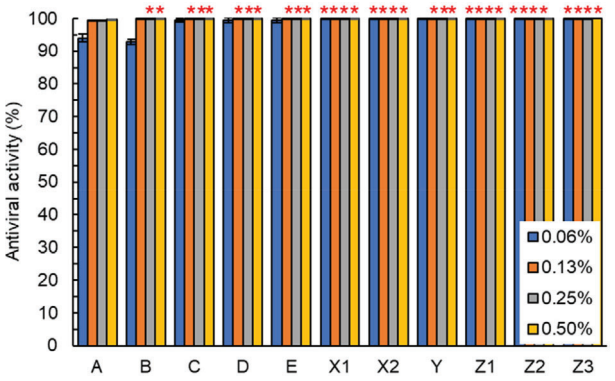


Figure 3. P22 inactivation activity of polyionenes within 30 s at their respective concentrations w/v%. The average limit of detection = 99.99%. Values equal to or above the limit of detection are indicated by the red asterisks. The experiments were performed in triplicates. Results were expressed as the mean antiviral activity ± standard deviation shown by the error bars (mean ± SD).

PHMB (Formulation **8**). Collectively, the polyionene was effective in killing bacteria and fungi in the presence of the thickener and the anionic surfactants, with or without alcohol.

Similar levels of antimicrobial activity were measured for X1, X2, Y, Z1, Z2, and Z3 (Table S3, Supporting Information). There were no colonies observed, which implied that there was at least 3-log reduction in viable bacteria within 30 s at the polyionene concentration of 0.5 w/v%.

2.4. Antiviral Activity of the Polyionenes Against the Nonenveloped Model Virus Bacteriophage Salmonella Virus P22

We first tested the antiviral activity of the polyionenes against the model enveloped virus, bacteriophage Salmonella virus P22. For this study, the polyionenes were prepared in water to reduce the possible interactions of the polyionenes with the protein components in the culture media. **Figure 3** shows the antiviral activity of various polyionenes for a contact time of 30 s. Polyionene **A** reduced the number of plaque-forming units by 2.7-log and 3-log at 0.25 and 0.5 w/v%, resulting in 99.4% and 99.7% antiviral activity, respectively. Polyionenes **B**, **C**, **D**, and **E** were even more effective and reduced the number of plaque-forming units by more than 4-log at 0.25 w/v% **B**) or at 0.125 w/v% **C**–**E**), resulting in greater than 99.99% antiviral activity. For polyionene **X1**–**Z3**, their antiviral activities were more than 99.9% even at 0.063 w/v% (more than 99.99% at 0.125 w/v% and above). These results demon-

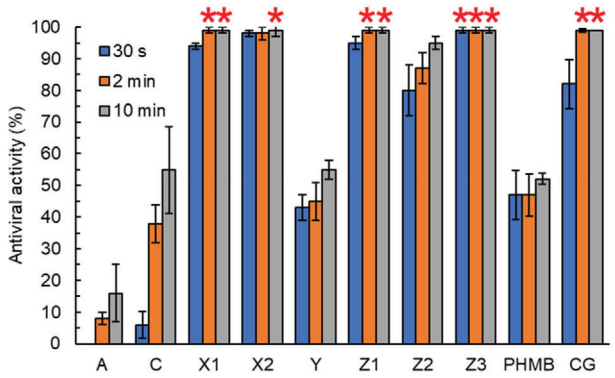


Figure 4. MHV antiviral activity of polyionenes, PHMB and chlorhexidine gluconate (CG) at 0.5 w/v% after 30 s, 2 min, or 10 min contact time. The limit of detection was 99%. Values equal to or above the limit of detection are indicated by the red asterisks. The experiments were performed in triplicates. Results were expressed as the mean antiviral activity ± standard deviation shown by the error bars (mean ± SD).

strate that increasing hydrophobicity increased the antiviral activity. Additionally, the polyionenes had higher antiviral activity against P22 than polyhexamethylene biguanide (only 98.3% at 0.5 w/v% in Table S4, Supporting Information). Chlorhexidine gluconate was not effective at inactivating the P22 bacteriophage (Table S4, Supporting Information).

2.5. Antiviral Activity Against the Enveloped Model Virus Mouse Coronavirus (MHV)

The polyionenes were next evaluated against MHV (**Figure 4**). Similarly, for this study, the polyionenes were prepared in water to reduce the possible interactions of the polyionenes with the protein components in the culture media. When polyionene **A** at 0.5 w/v% was in contact with the MHV for 2 min, it deactivated 8% of the initial viral loading. Increasing the concentration of polyionene **A** by eightfolds increased the antiviral activity from 8% to 41% (Table S5, Supporting Information). Similarly, increasing the concentration of polyionene **C** by eightfolds increased the antiviral activity from 38% to 80%. Increasing the contact time to 10 min had a smaller effect as there was no significant increase in the antiviral activity. In contrast, the more hydrophobic polyionenes had higher antiviral activity at 0.5 w/v%. Of these five polyionenes, **Y** which has the least hydrophobic linker inactivated 43% and 55% of MHV within 30 s and 10 min, respectively. For polyionenes **X1** and **X2**, the substitution of the hydrogen with the methyl group resulted in slightly higher antiviral activity within 30 s, antiviral activity for **X1** and **X2** were 94% and 98%, respectively. After 2 min of incubation, the antiviral activities of **X1** and **X2** were close to the limit of detection of 99%. Compared to **Z1**, **Z2** was less hydrophobic due to the introduction of less hydrophobic monomer R1 (Scheme 1 **III**). The introduction of rigid monomer **R2** into **Z3** did not compromise antiviral activity, and stronger antiviral activity was observed especially for 30 s of incubation, resulting in 99% antiviral activity (the limit of detection). Notably, these hydrophobic polyionenes had higher antiviral activity than polyhexamethylene biguanide. Polyionenes **X1** and **Z1** were comparable in antiviral activity to chlorhexidine gluconate, while **Z3** had stronger activity against the enveloped virus as compared to chlorhexidine gluconate.

Table 6. Inhibition of SARS-CoV-2 replication by 30 s of treatment with polyionenes **A**, **X2**, and **Z3** at 0.1 and 1 w/v%. Results were expressed as the mean antiviral activity ± standard deviation from two independent experiments.

Polyionene	Concentration [w/v%]	30 s
A	0.1	99.9996 ± 0.0001
	1	99.999 748 ± 0.000 002
X2	0.1	99.99 997 ± 0.00 001
	1	99.99 996 ± 0.00 003
Z3	0.1	99.99 996 ± 0.00 001
	1	99.99 985 ± 0.00 003

Table 7. The median lethal doses (LD50) of polyionenes when administered orally to mice. The maximum dose tested was 1750 mg kg^{-1} as the polymers were not soluble in water at higher concentrations.

Polyionene	LD50 [mg kg^{-1}]
A	1323
B	1684
C	1684
D	1323
E	1684
X1	>1750
X2	>1750
Z1	>1750
Z2	>896[a)]
Z3	>896[a)]
Polyhexamethylene biguanide	434
Chlorhexidine digluconate	2500[b)]

[a)] This is the highest concentration of the polyionene that could be dissolved in water
[b)] Extracted from. [14]

2.6. Antiviral Activity Against SARS-CoV-2

To evaluate if the polyionenes are able to inactivate SARS-CoV-2, the inhibition of SARS-CoV-2 replication by 30 s of treatment with the polyionenes **A**, **X2**, and **Z3** was quantified using quantitative real-time PCR using the N gene and ORF1ab gene as probes (**Table 6**). At 0.1 w/v% and 30 s contact time, polyionenes **A**, **X2**, and **Z3** inactivated the virus, and the virus replication was inhibited by at least 99.999%. There was no significant difference in activity among the three polyionenes and between polyionene concentrations at 0.1 and 1 w/v%.

2.7. Evaluation of In Vivo Compatibility

For polyionenes **A–E**, the median lethal dose (LD50) by oral administration ranged from 1323 to 1684 mg kg^{-1} (**Table 7**). For polyionenes **Z2** and **Z3**, the highest dose tested was 896 mg kg^{-1} because this was the maximum concentration they could be dissolved in water. At this dose, the mice survived with no apparent

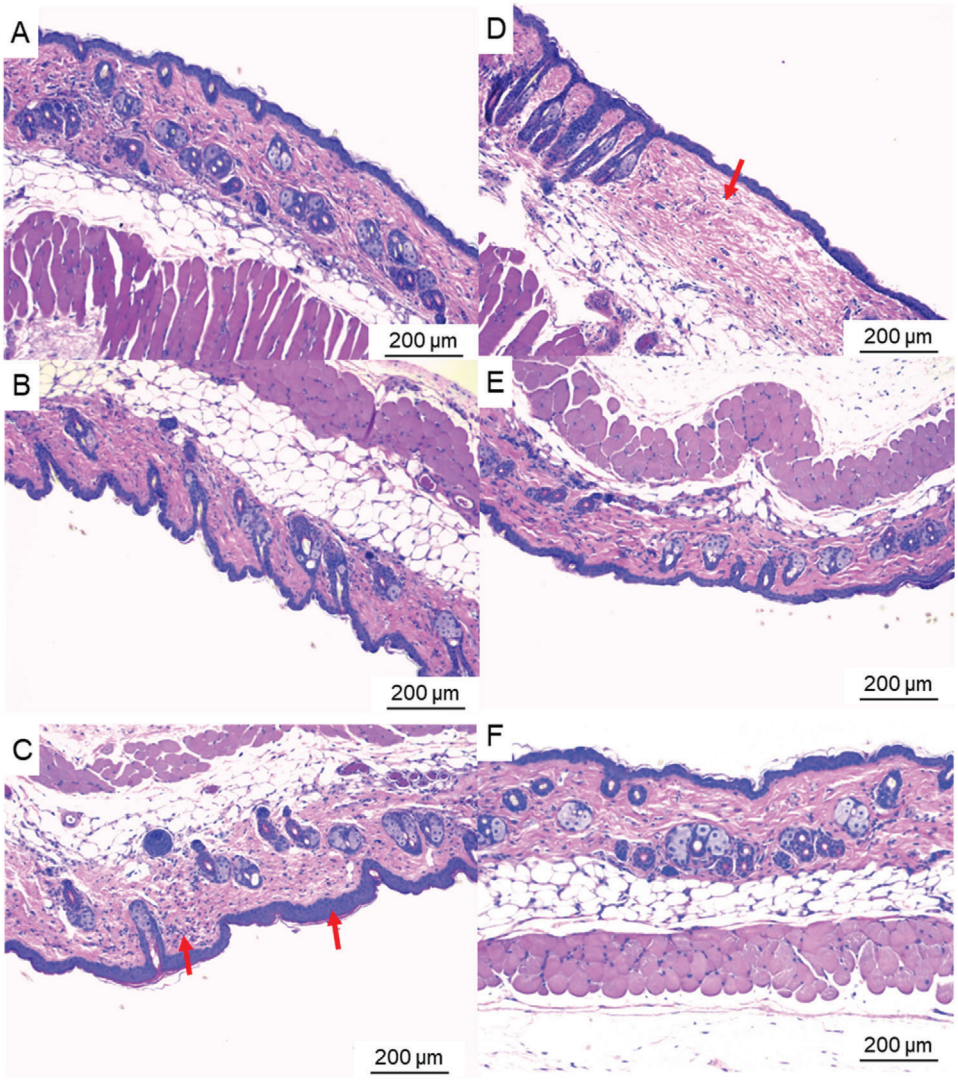


Figure 5. Histological examination of the mouse skin after topical treatments using A) saline, B) alcohol-containing commercial hand sanitizer, C) nonalcohol-containing hand sanitizer containing 4 w/v% chlorhexidine gluconate, D) polyionene A (0.5 w/v%) prepared in alcohol-containing formulation, E) polyionene A (0.5 w/v%) prepared in nonalcohol-containing hand sanitizer formulation, F) polyionene Z1 (0.5 w/v%) prepared in nonalcohol-containing hand sanitizer formulation.

toxicity. For polyionenes **X1**, **X2**, and **Z1**, mice survived with no apparent toxicity at 1750 mg kg^{-1}. These polyionenes were not tested at doses higher than 1750 mg kg^{-1} as they were no longer soluble in water. The LD50 of the polyionenes were higher than that of polyhexamethylene biguanide, which was 434 mg kg^{-1}.

To evaluate the safety of their use as a hand sanitizer or hand wash, polyionenes **A** and **Z** were tested for skin compatibility on mice (**Figure 5**). The skin on the backs of mice was treated topically with one of the six treatments: A) control (saline), B) alcohol-containing commercial hand sanitizer (62 w/w% ethanol, 3 w/w% isopropanol, 1% niacinamide in a gel base), C) nonalcohol-containing commercial hand wash (4 w/v% chlorhexidine gluconate as the active ingredient, poloxamer 237, isopropyl alcohol, lauryl dimethyl amine oxide, glycerol, macrogol 7 glycerol cocoate, gluconolactone, perfume (Herbacol), ponceau 4R (E124), sodium hydroxide, and purified water), (D) polyionene **A** prepared in alcohol-containing formulation (30% ethanol, 0.5 w/v% polyionene **A** and 1% HPMC), (E) polyionene A (0.5 w/v%) in a nonalcohol-containing formulation, (F) polyionene Z1 (0.5 w/v%) in the same nonalcohol-containing formulation. The nonalcohol-containing formulation consists of 1 w/v% HPMC, 5 w/v% octylphenoxy polyethoxyethanol, and 5 w/v% decyl *β-D*-glucopyranoside, which are known to be nontoxic. For the hand sanitizer formulations, the skin was treated for 4 h each time, twice a day for 4 consecutive days. For the hand wash formulations, the skin was treated for 2 min each time, 4 times a day for 4 consecutive days. The skin samples were evaluated for any compromise in the integrity of the epidermis, changes in tissue structure, or evidence of inflammation in the epidermis, dermis, and subcutaneous levels. The high alcohol content in the commercial hand sanitizer formulation did not cause obvious adverse side-effects to the skin as compared to the control group (Figure 5A,B).

Like the skin treated with the commercial hand sanitizer formulation, there was no evidence of compromise in the superficial layer with few inflammatory cells in the samples representing the polyionene A and polyionene Z1-treated skin. While there was mild accumulation of fluid in the dermis (arrow in Figure 5D), this was not pathological. Importantly, the overall structure and cellular integrity was preserved.

In contrast, the skin of the mice that were treated with a nonalcohol-containing commercial hand wash containing 4 w/v% chlorhexidine gluconate showed a thickened keratin layer (arrows in Figure 5C). There were also signs of epidermal cell proliferation and recruitment of several inflammatory cells in the dermal layer.

3. Discussion

A series of polyionenes was synthesized and evaluated for antimicrobial and antiviral activities. The polyionenes were synthesized by condensation-type polymerization, with the quaternary ammonium groups installed on the backbone of the polymer. The hydrophobicity gradually increased from polyionene **A** to polyionene **E** due to the increasing content of the monomer **c** in Scheme 1. Polyionenes **X1–Z3** represent a further increase in hydrophobicity by using the more hydrophobic monomers **d**, **e**, **g**, **h**, or **R2** as represented in Scheme 1.

The antimicrobial mechanism of cationic polymers is by membrane-disruption of the microbial cells, which led to broad spectrum activity.[13] Therefore, the polyionenes made in this study were tested against an assortment of common opportunistic human pathogens, such as *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans*. Although polyionene A–E contained an increased amount of the relatively hydrophobic monomer **c**, there was no significant difference in MIC and MBC especially against the Gram-negative bacteria tested. Employing the more hydrophobic monomers **d**, **e**, **g**, **h**, and **R2** to make polyionenes **X1–Z3** increased MIC and MBC/MFC values in all bacteria and fungi tested as compared to polyionenes **A–E**. This phenomenon was also observed in other polymer systems.[15] The relatively more hydrophobic polyionenes might interact with the proteins present in the culture medium, masking their antimicrobial activity. Consistently, we observed that media containing polyionenes **X1**, **X2**, **Y**, **Z1**, **Z2**, and **Z3** were cloudier with micrometer-sized aggregates than that of polyionenes **A–E** especially at high concentrations. By comparing the MIC and the HC50 values, polyionenes **A–E** had excellent selectivity toward the bacteria and fungi than the mammalian cells. This could be due to the differences between the bacterial and mammalian cell membrane compositions where the bacterial membranes have a net negative charge and mammalian cell membranes have a net neutral charge. Polyionenes **X1**, **X2**, **Y**, **Z1**, **Z2**, and **Z3** had relatively low selectivity toward the bacteria and fungi. This is similar to previous observations where hydrophobic macromolecules would preferentially form hydrophobic interactions with the lipid layer of mammalian cells that have a net neutral charge.[16]

As enzymes and proteins involved in bacterial drug resistance have no effect on the polyionenes, the polyionenes were just as effective against drug-resistant bacteria. The MICs and MBCs for polyionenes **A** and **C** were very similar to those of patient-derived MRSA and NDM-producing *K. pneumoniae* (Table 2). Additionally, in contrast to antibiotics, the membrane-disrupting mechanism is not dependent on the microorganism metabolism or proliferation. As a result, the polyionenes were also fast acting. When tested in hand sanitizer/wash formulations, polyionene A showed more than 99.9% bactericidal and fungicidal activity for a contact time of 30 s. In both formulations with and without ethanol, there was more than 99.9% bactericidal activity. This indicates that the thickening agents or surfactants do not inhibit the interactions of the polyionenes with the bacteria.

In the context of the COVID-19 pandemic, we were also interested to determine if the polyionenes were able to deactivate viruses. The polyionenes were very effective in deactivating the nonenveloped model virus bacteriophage P22. The highly charged polymers could act as chaotropic agents to interfere with the viral proteins and in turn, deactivated them to prevent them from infecting and replicating. As a surrogate for the SARS-CoV-2, we tested the polyionenes against the murine hepatitis virus (MHV). The MHV is a member of the *Coronaviridae* family and has several structural similarities to SARS-CoV-2.[17] For example, spike proteins populate the surface of the viral envelope. However, a distinct difference is that the MHV has a viral envelope thickness of about 7.6 nm, which is thicker than the SARS-CoV2 membrane of 3.9 nm.[18] Despite the thick lipid membrane, the polyionenes prevented viral infection to mammalian cells by inactivating the viruses prior to cell contact. There are negatively charged regions in the membrane proteins of virus, and the cationic polyionenes might interact with the proteins through electrostatic interaction and thus mask the virus, preventing it from infecting cells.[19] Another possible mechanism is that the hydrophobic components of the polymers disrupted the viral membrane, inactivating the virus. This could be the reason why polyionene **Z3**, with the longest hydrocarbon alkyl groups and the rigid monomer 4,4'-bis(chloromethyl)biphenyl, was the most effective in deactivating the MHV. Interestingly, when polyionenes A, X2, and Z3 were tested against SARS-CoV-2, all three had similar viral inhibition activity. All three were very effective against SARS-CoV-2 and the activity was independent of the polyionene hydrophobicity. As discussed above, this could be due to the SARS-CoV-2's thinner lipid envelope. As a result, the relatively less hydrophobic polyionene A was more effective against the SARS-CoV-2 than MHV in disrupting the viral envelope and inhibiting viral infection and replication.

The polyionenes were compatible to healthy mouse skin. Unlike small molecules, which have a molecular size of 500 g mol^{-1}, can penetrate the skin, [7] the polyionenes with relatively large molecular sizes (M_w >10 000 g mol^{-1}) are unable to enter the skin. This is advantageous for the polyionenes as there could be long term adverse effects derived from repeated exposure to the small molecules. In addition, as an estimate of acute toxicity, the oral median lethal doses (LD50) of the polyionenes in mice are more than 1 g kg^{-1}, demonstrating that they are relatively safer to handle than the commonly used disinfectants, such as benzalkonium chloride (0.242 g kg^{-1} in mice)[20] and polyhexamethylene biguanide (0.434 g kg^{-1} in mice). In the control skin compatibility study, the signs of inflammation from the repeated treatments with chlorhexidine were observed on the skin (Figure 5). This phenomenon was also reported in other papers, plus allergic reactions and even low concentrations of chlorhexidine gluconate in the blood of some individuals after repeated use.[20–21]

4. Conclusion

A series of new polyionenes were designed, synthesized and characterized as antimicrobials and antivirals. These polyionenes were effective against a broad spectrum of bacteria, fungi and MDR strains of bacteria. There was no compromise in antimicrobial activity when formulated with thickener and nonionic surfactants. They had potent activity against the nonenveloped virus, independent of hydrophobic components while only polyionenes **X** and **Z**, which were made from the more hydrophobic monomers **d** and **e** (containing two cyclic hexyl groups) as well as **h** (containing a long alkyl C12 group), demonstrated excellent activity against the enveloped virus MHV. Particularly, among the polyionenes reported in this study, Z3 exhibited the strongest activity against MHV. Importantly, the polyionenes were more effective against SARS-CoV-2 than MHV, and inhibited SARS-CoV-2 replication by >99.999%. Their antiviral activity against both the enveloped and the nonenveloped virus types was stronger than the commercial antimicrobial agents PHMB and chlorhexidine gluconate. These polyionenes were safe to handle with high oral LD50 values in mice and showed compatibility to mouse skin when used as hand wash or hand sanitizer.

5. Experimental Section

Materials: All chemical reagents were provided by Sigma-Aldrich or Tokyo Chemical Industry (TCI) and used as received unless specified otherwise. Potentially biodegradable monomers with two benzyl chloride groups (**c**, **d**, **e**, **g**, and **h**) were synthesized using the same or similar protocol, which we reported previously.[13b,c]

Staphylococcus aureus (ATCC 6538), *Escherichia coli* (ATCC 25 922), *Pseudomonas aeruginosa* (ATCC 9027), and *Candida aureus* (ATCC 10 231) were purchased from ATCC and reconstituted based on the recommended protocols. NCTC-1469 cells were purchased from CLS Cell Lines Service GmbH and maintained in a 37 °C, 5% CO_2 incubator, in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% horse serum and 1% penicillin-streptomycin. *Salmonella enterica* serovar Typhimurium (ATCC 14 028) and bacteriophage P22 were kind donations from Genome Institute of Singapore. MHV-A59 was a kind donation from Yee Joo Tan (Institute of Molecular and Cell Biology, Singapore). Mueller Hinton broth (MHB) was purchased from BD Diagnostics (Singapore) and was prepared according to the manufacturer's instructions.

Synthesis of Cationic Polyionenes: The experimental procedures for synthesis of the polyionenes are similar to that reported in an earlier publication[13b] and the synthesis of polyionene **Z3** was given below as a typical example. Briefly, in a 500 mL of blue cap bottle equipped with a magnetic stir bar, **h** (1.01 g, 2 mmol), and 4,4"-Bis(chloromethyl)biphenyl (0.53 g, 2 mmol) were suspended in *N*-methylpyrrolidone (NMP, 10 mL) and the solution heated to 80 °C to render a clear solution. *N,N,N",N'*-Tetramethyl-1,6-diaminohexane (**f**, 0.88 mL, 4 mmol) was added under stirring and the solution was allowed to stir at 80 °C overnight. The reaction mixture was cooled down to room temperature and 200 mL of diethyl ether was poured to precipitate the crude product, which was purified by vacuum filtration and washed with diethyl ether for three times. The isolated solid was dried in vacuo to result in white solid at near quantitative yields. Finally, the solid was further purified by first dissolving in de-ionized (DI) water, followed by extensive dialysis against DI water using dialysis membrane with a molecular weight cut-off (MWCO) of 1 kDa, and lyophilization to result in the target polymer as white powdery solid. Yield, 1.44 g, 62%; Mn 10400, PDI 1.20. ^{1}H NMR (400 MHz, MeOD, 22 °C): δ 7.96 (d, 4nH, –Ph*H*–), 7.87 (d, 4nH, –Ph*H*–), 7.72 (t, 8nH, –Ph*H*–), 4.64 (s, 8nH, –PhCH_2–), 3.39 (m, 12nH, –C(O)NHCH_2– and –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 3.09 (d, 24nH, –$N^{\oplus}(CH_3)_2$–), 2.00 (s, br, 8nH, –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 1.48–1.68 (m, 12nH, –C(O)NHCH$_2$CH_2– and –CH_2CH_2– of **f**), 1.34 (m, 16nH, – $(CH_2)_8$– of **h**).

A: Yield, 75%. Mn: 7930; PDI: 1.34. ^{1}H NMR (400 MHz, D_2O, 22 °C): δ 7.78 (dd, 10nH, –Ph*H*– of **c**), 7.68 (s, 70nH, –Ph*H*– of **b**), 7.62 (dd, 10nH, –Ph*H*– of **c**), 4.60 (s, 80nH, –PhCH_2–), 3.59 (s, 10nH, –CH_2CH_2– of **c**), 3.25 (s, 80nH, –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 3.07 (s, 240nH, –$N^{\oplus}(CH_3)_2$), 2.53 (s, br, 40nH, –$CH_2CH_2CH_2$– of **a**).

B: Yield, 73%. Mn: 7450; PDI: 1.38. ^{1}H NMR (400 MHz, D_2O, 22 °C): δ 7.78 (t, 20nH, –Ph*H*– of **c**), 7.67 (s, 60nH, –Ph*H*– of **b**), 7.60 (t, 20nH, –Ph*H*– of **c**), 4.60 (s, 80nH, –PhCH_2–), 3.59 (s, 20nH, –CH_2CH_2– of **c**), 3.47 (m, 80nH, –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 3.07 (s, 240nH, –$N^{\oplus}(CH_3)_2$), 2.51 (s, br, 40nH, –$CH_2CH_2CH_2$– of **a**).

C: Yield, 70%. Mn: 6750; PDI: 1.41. ^{1}H NMR (400 MHz, D_2O, 22 °C): δ 7.77 (m, 40nH, –Ph*H*– of **c**), 7.66 (m, 40nH, –Ph*H*– of **b**), 7.58 (m, 40nH, –Ph*H*– of **c**), 4.59 (m, 80nH, –PhCH_2–), 3.59 (s, 40nH, –CH_2CH_2– of **c**), 3.25 (s, 80nH, –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 3.06 (s, 240nH, –$N^{\oplus}(CH_3)_2$), 2.48 (s, 40nH, –$CH_2CH_2CH_2$– of **a**).

D: Yield, 71%. Mn: 6180; PDI: 1.50. ^{1}H NMR (400 MHz, D_2O, 22 °C): δ 7.75 (d, 60nH, –Ph*H*– of **c**), 7.65 (m, 20nH, –Ph*H*– of **b**), 7.57 (d, 60nH, –Ph*H*– of **c**), 4.55 (m, 80nH, –PhCH_2–), 3.58 (s, 60nH, –CH_2CH_2– of **c**), 3.38 (m, 80nH, –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 3.04 (s, 240nH, –$N^{\oplus}(CH_3)_2$), 2.40 (s, br, 40nH, –$CH_2CH_2CH_2$– of **a**).

E: Yield, 71%. Mn: 6550; PDI: 1.46. ^{1}H NMR (400 MHz, D_2O, 22 °C): δ 7.76 (dd, 70nH, –Ph*H*– of **c**), 7.65 (s, 10nH, –Ph*H*– of **b**), 7.57 (dd, 70nH, –Ph*H*– of **c**), 4.55 (s, 80nH, –PhCH_2–), 3.58 (s, 70nH, –CH_2CH_2– of **c**), 3.38 (m, 80nH, –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 3.03 (s, 240nH, –$N^{\oplus}(CH_3)_2$), 2.43 (s, br, 40nH, –$CH_2CH_2CH_2$– of **a**).

X1: Yield, 75%; Mn 5770, PDI 1.62; ^{1}H NMR (400 MHz, CD_3OD, 22 °C): δ 7.94 (m, 4nH, –Ph*H*–), 7.69 (d, 4nH, –Ph*H*–), 4.63 (s, 4nH, –PhCH_2–), 3.92 (m, 2nH, -–CONHC*H*–), 3.41 (m, 4nH, –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 3.08 (s, 12nH, –$N^{\oplus}(CH_3)_2$–), 1.02–2.04 (m, 26nH, –CH_2– of **d** and –$(CH_2)_4$– of **f**).

X2: Yield, 72%; Mn 5800, PDI 1.57; ^{1}H NMR (400 MHz, CD_3OD, 22 °C): δ 7.94 (m, 4nH, –Ph*H*–), 7.70 (d, 4nH, –Ph*H*–), 4.63 (s, 4nH, –PhCH_2–), 3.59 (m, 2nH, –CONHC*H*–), 3.41 (m, 4nH, –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 3.08 (s, 12nH, –$N^{\oplus}(CH_3)_2$–), 0.76–2.04 (m, 30nH, –CH_2– and H of cyclohexyl of **e**, and –$(CH_2)_4$– of **f**).

Y: Yield, 68%; Mn 10870, PDI 1.16; ^{1}H NMR (400 MHz, CD_3OD, 22 °C): δ 7.96 (dd, 4nH, –Ph*H*–), 7.70 (dd, 4nH, –Ph*H*–), 4.64 (s, 4nH, –PhCH_2–), 3.41 (m, 8nH, –C(O)NHCH_2– and –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 3.08 (s, 12nH, –$N^{\oplus}(CH_3)_2$–), 1.98 (s, br, 4nH, –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 1.66 (m, 4nH, –C(O)NHCH$_2$CH_2–), 1.40–1.58 (m, 8nH, –CH_2CH_2– of **f** and **g**).

Z1: Yield, 65%; Mn 5990, PDI 1.45; ^{1}H NMR (400 MHz, CD_3OD, 22 °C): δ 7.95 (dd, 4nH, –Ph*H*–), 7.70 (dd, 4nH, –Ph*H*–), 4.63 (s, 4nH, –PhCH_2–), 3.38 (m, 8nH, –C(O)NHCH_2– and –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 3.08 (s, 12nH, –$N^{\oplus}(CH_3)_2$–), 1.97 (m, 4nH, –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 1.63 (m, 4nH, –C(O)NHCH$_2$CH_2–), 1.52 (m, 4nH, –CH_2CH_2– of **f**), 1.31 (m, 16nH, –$(CH_2)_8$– of **h**).

Z2: Yield, 70%; Mn 8760, PDI 1.30; ^{1}H NMR (400 MHz, CD_3OD, 22 °C): δ 7.96 (dd, 4nH, –Ph*H*–), 7.78 (s, 4nH, –Ph*H*–), 7.71 (dd, 4nH, –Ph*H*–), 4.65 (m, 8nH, –PhCH_2–), 3.39 (m, 12nH, –C(O)NHCH_2–, and –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 3.09 (d, 24nH, –$N^{\oplus}(CH_3)_2$–), 1.99 (s, br, 8nH, –$CH_2CH_2N^{\oplus}(CH_3)_2$–), 1.49–1.68 (m, 12nH, –C(O)NHCH$_2$CH_2–, and –CH_2CH_2– of f), 1.34 (m, 16nH, – $(CH_2)_8$– of h).

MIC, MBC, and Hemolysis Measurements and Membrane Permeability Analysis Using a Confocal Laser Scanning Microscope: The MIC and MBC of polyionenes was determined against 4 clinically relevant microbes: *S. aureus* (ATCC 29 737), *E. coli* (ATCC 25 922), *P. aeruginosa* (ATCC 9027), and *C. albicans* (ATCC 10 231), using the previously reported broth microdilution method.[13] The hemolysis assay was conducted using the previously reported method.[13] The experiments were performed in triplicates. The preparation of bacterial cells for imaging was conducted using a previously reported method.[23] The images were taken using a Zeiss LSM710 confocal microscope.

Antimicrobial Efficiency of Hand Sanitizer (with Alcohol) and Hand Wash (without Alcohol) Formulations: Eight formulations of hand sanitizers were prepared with different components in DI water: 1) 1 w/v% hydroxypropyl)methyl cellulose (HPMC); 2) 1 w/v% HPMC + 0.5 w/v%

polyionene A; 3) 1 w/v% HPMC + 0.5 w/v% polyionene A + 30 w/v% pure alcohol; 4) 1 w/v% HPMC + 0.5 w/v% polyionene A + 50 w/v% pure alcohol; 5) 1 w/v% HPMC, 5 w/v% octylphenoxy polyethoxyethanol, 0.5 w/v% polyionene A, 5 w/v% decyl β-D-glucopyranoside; 6) 1 w/v% HPMC, 5 w/v% octylphenoxy polyethoxyethanol, 0.5 w/v% polyionene A, 5 w/v% *N,N*-bis(2-hydroxyethyl)dodecanamide; 7) 4 w/v% chlorhexidine; 8) 0.004 w/v% polyhexamethylene biguanide (PHMB). The killing efficiencies of the eight hand sanitizer formulations were determined against *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans*. In brief, the bacteria suspension was prepared at the final concentration of 5×10^6 CFU mL^{-1}. Subsequently, 20 µL of the bacteria suspension was added to 1 mL of hand sanitizer/wash solution (except for formulation 1) and vortexed for 30 s before plating with 250 µL on each agar plate. For the control group (1 mL of MHB) and formulation 1, 20 µL of the bacteria suspension was added to 1 mL of MHB or formulation 1, then the mixture was further diluted 100-folds by taking 20 µL and adding to 2 mL of MHB (final concentration: 10^3 CFU mL^{-1}), followed by plating with 100 µL on each agar plate. For *S. aureus*, *P. aeruginosa*, and *E. coli*, the agar plates were incubated overnight in the 37 °C incubator. For the *C. aureus*, the agar plates were incubated for 48 h at room temperature. The experiments were performed in triplicates.

Antiviral Activity Against the Nonenveloped Model Virus Bacteriophage Salmonella P22 Virus: The polymers were dissolved in DI water (1 mL) at concentrations of 0.125, 0.25, and 0.5 w/v%. Bacteriophage suspension (10 µL, $\approx 10^8$ PFU mL^{-1}) was added into the polymer solutions and vortexed for 30 s. After 30 s, 50 µL of the bacteriophage and polymer mixture were diluted in 500 µL of phosphate-buffered saline (PBS). The polymers were separated from the bacteriophage using a centrifugal filter (MWCO 50 000) and the samples were centrifuged at 1800×*g*, 10 min at 4 °C. The mixtures were washed 2 times by adding 480 µL of PBS after each centrifugation. At the end of the 3rd centrifugation, the bacteriophages were collected and MHB was added to make up the volume to 1 mL. The concentrations of bacteriophages were quantified by adding 50 µL of overnight culture of *Salmonella enterica* serovar Typhimurium to 5 mL of molten soft agar (0.75% agar in LB broth) followed by adding 50 µL of the bacteriophage suspension and pouring onto solid 1.5% agar plates. After 18 h, visible plagues were counted and the viral titer was calculated by multiplying the average number of plaques per well by the serial dilution value to determine the virus concentration in 1 mL of the assayed solution.[22] Results were expressed as plaque-forming units (PFU) mL^{-1}. The antiviral activity = ([viral titer in water]-[viral titer in polymer solution])/[viral titer in water] × 100%. The experiments were performed in triplicates. Results were expressed as the mean antiviral activity ± standard deviation shown by the error bars (mean ± SD).

Antiviral Activity Against the Model Enveloped Virus (Mouse Coronavirus, MHV): The polymers were dissolved in DI water (1 mL) at concentrations of 0.125, 0.25, and 0.5 w/v%. MHV-A59 suspension (10 µL, $\approx 10^8$ PFU mL^{-1}) was added into the polymer solutions and vortexed for 30 s. After 30 s, 50 µL the MHV and polymer mixtures were diluted in 500 µL of PBS. The polymers were separated from the MHV using a centrifugal filter (MWCO 50000), and were centrifuged at 1800×*g*, 10 min at 4 °C. The mixtures were washed 2 times by adding 480 µL of PBS after each centrifugation. At the end of the 3rd centrifugation, the MHV particles were collected and DME2 (DMEM supplemented with 2% FBS) was added to make up the volume to 1 mL. The active viral titers were quantified by a plaque assay. NCTC 1469 cells were the host for the plaque assay. To perform the assay, 1.2 million cells were seeded overnight on a 6-well plate. Prior to viral adsorption, the cells were washed with PBS. Then 500 µL of viral sample was incubated with the cells for 1 h at 37 °C with intermittent shaking. After 1 h, a warm mixture of DME2 and agarose (final concentration: 0.8 w/v%) was added to each of the wells. The well plates were incubated at 37 °C for 2 days before they were fixed with neutral buffered formalin and stained with crystal violet. The viral titer was calculated by multiplying the average number of plaques per well by the serial dilution value to determine the virus concentration in 1 mL of the assayed solution. Results were expressed as plaque-forming units (PFU) mL^{-1}.[23] The antiviral activity = ([viral titer in water]-[viral titer in polymer solution])/[viral titer in water] × 100%. The experiments were performed in triplicates. Results were expressed as the mean antiviral activity ± standard deviation shown by the error bars (mean ± SD).

Antiviral Activity Against SARS-CoV-2: The polymers were dissolved in DI water (0.5 mL) at concentrations of 0.1 and 1 w/v%. SARS-CoV-2 suspension (50 µL, $\approx 10^9$ $TCID_{50}$ mL^{-1}, hCoV-19/Hangzhou/ZJU-05/2020, GISAID Accession ID: EPI_ISL_415 711) [24] was added into the polymer solutions and vortexed for 30 s. After 30 s, 10 µL of the SARS-CoV-2 and polymer mixtures were diluted and mixed thoroughly in 1 mL of PBS. 200 µL of the mixture were added to each well of a 12 well plate followed by 1.8 mL of Vero cells (ATCC CCL-81) suspended (10^5 cells mL^{-1}) in MEM supplemented with 5% FBS. After incubating at 35 °C in 5% CO_2 for 6 days, the culture supernatants were collected for nucleic acid extraction. The viral nucleic acid abundance was measured using SARS-CoV-2 qRT-PCR kits (DaAn Gene, Guangzhou, China), targeting the ORF1ab and N genes. The percentage of inhibition was calculated as: Inhibition (%) = $(1–2^{-\Delta Ct}) \times 100\%$. All experiments involving SARS-CoV-2 were conducted in an approved biosafety level III laboratory (CNAS BL0022, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Zhejiang University).

Determination of the Median Lethal Dose (LD50) of the Polyionenes by Oral Administration: All animal experiments were conducted in accordance with the approved protocol from the Institutional Animal Care and Use Committee (IACUC) at the A*STAR Biological Resource Centre of Singapore. The LD50 experiments were conducted on female Balb/c mice (8–12 weeks old, 18–22 g). The Up-and-Down procedure described in OECD Guidelines for the Testing of Chemicals (OECD 425) was used as a guide. Polyionenes were dissolved in water and administered to the mice using an oral gavage at various doses (896, 1120, 1400, 1750 mg kg^{-1}, 0.2–0.4 mL). The survival rate was monitored for 14 days post-treatment, and the LD50 was estimated using the maximum likelihood method.[25]

Skin Compatibility Study: All animal experiments were conducted in accordance with the approved protocol from the Institutional Animal Care and Use Committee (IACUC) at the A*STAR Biological Resource Centre of Singapore. The in vivo skin irritation study was conducted on female Balb/c mice (8–12 weeks old, 18–22 g). Before the test, the backs of the mice were shaved to expose an area of at least 2 × 2 cm of skin. The shaver was first used to shorten the hair before fully removing the fur with hair removal cream. The mice were randomly grouped into 6 groups, with 6 mice in each group. The 5 treatment groups were: 1) Control (saline), 2) Alcohol-containing commercial hand sanitizer (62 w/w% ethanol, 3 w/w% isopropanol, 1% niacinamide in a gel base), 3) Polyionene A prepared in alcohol-containing formulation (30% ethanol, 0.5 w/v% polyionene A, and 1% HPMC), 4) Nonalcohol-containing commercial hand wash containing 4 w/v% chlorhexidine gluconate as the active ingredient and the following excipients: poloxamer 237, isopropyl alcohol, lauryl dimethyl amine oxide, glycerol, macrogol 7 glycerol cocoate, gluconolactone, perfume (Herbacol), ponceau 4R (E124), sodium hydroxide, and purified water, 5) Polyionene A prepared in a nonalcohol-containing hand wash formulation (0.5 w/v% polyionene A, 1% HPMC, 5% octylphenoxy polyethoxyethanol and 5 w/v% decyl β-D-glucopyranoside), and 6) Polyionene Z1 prepared in a nonalcohol-containing hand wash formulation (0.5 w/v%, 1 w/v% HPMC, 5 w/v% octylphenoxy polyethoxyethanol, and 5 w/v% decyl β-D-glucopyranoside). The treatments were applied onto the skin using a paintbrush. For groups 1–3, a thick coat was applied onto the shaved backs and were left for 4 h. Then, the skin was washed with water and dried 4–5 times until the backs were clean. A new layer of the sample was then applied. This process was repeated twice a day for 4 consecutive days. For groups 4 and 5, a coat was applied onto the treated backs and left on the skin for 2 min. Then, the skin was washed with water and dried 4–5 times until the backs were clean. After 2 h, the sample was applied and a new coat was used to cover the treated skin. This process was repeated 4 times a day for 4 days. After the last treatment, the mice were sacrificed and the skin tissues were harvested for histological examination. The skin samples were fixed with 4% neutral buffered formalin and placed in paraffin blocks. The samples were sectioned and stained with hematoxylin and eosin using standard protocols.

Statistical Analysis: Data are presented as mean ± standard deviation. To assess significant differences, Student's *t*-test was used, and the difference was considered statistically significant when $p < 0.05$.

Supporting Information

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

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data available on request from the authors.

Keywords

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