치의과학 박사학위논문

Gemini-Mediated Self-Disinfectant to Prevent Contact Transmission of Infectious Diseases

전염성 감염질환의 접촉 전파 방지를 위한 제미니형 자가 표면 항균제 연구

2022년 8월

서울대학교 대학원
치의과학과 치과생체재료과학 전공

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지도교수 안 진 수

이 논문을 치의과학 박사학위논문으로 제출함
2022년 5월

서울대학교 대학원
치의과학과 치과생체재료과학 전공
김 민 주

김민주의 박사학위논문을 인준함
2022년 7월

위 원 장 임 범 순 (인)

부 위 원 장 안 진 수 (인)

위 원 이 상 훈 (인)

위 원 박 지 만 (인)

위 원 김 종 은 (인)

Abstract

MinJu Kim

Department of Dental Biomaterials Science

Graduate School of Dentistry

Seoul National University

Directed by Professor Jinsoo Ahn, D.D.S., Ph.D.

Introduction: The primary transmission passage of infectious diseases is surface contact, yet no definitive prevention of contact transmission has been established. The current surface disinfecting materials are effective only at the time of application, and further contact with pathogens render re-contamination.

Methods: Three members of new gemini diquaternary organosilanes were synthesized to determine their efficacy as a traditional sanitizer. The ability to generate a self-disinfecting surface against an array of bacterial and viral pathogens were tested against *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Severe acute respiratory syndrome-coronavirus-2* (SARS-CoV-2). The newly

synthesized gemini diquaternary silane solutions were tested against microbes as suspension and coatings of both porous and non-porous surfaces. The solutions were further evaluated for contact angle measurements, mechanical and chemical durability, resistance against re-contamination, and cytotoxicity.

Results: Aqueous solutions of gemini compounds showed 99.9999% reduction of all tested bacteria and viruses in suspension and on the surfaces up to 15 days. Gemini-inspired disinfectants were demonstrated to suppress almost 99.9999% of microbial activity than conventional antimicrobial surface treatments when applied to porous and nonporous materials. The newly synthesized gemini disinfectants retained antimicrobial activity against mechanical and chemical stress, and re-contamination. They were less cytotoxic than conventional monoquaternary ammonium silane disinfectant.

Conclusion: The newly synthesized gemini-disinfectant possesses the same immediate activity of traditional disinfectants when initially applied to a surface, but concomitantly endows long-lasting antimicrobial activity whereby bacteria and viruses that subsequently settle upon it are continuously deactivated.

Keyword : fomite transmission, disinfectants, sanitizers, quaternary ammonium, gemini surfactants, antimicrobial
Student Number : 2018-34220

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1. Introduction

1.1. Study Background

Infectious diseases are directly responsible for more than 20% global deaths annually. According to the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC), infectious diseases including Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are primarily transmitted between people or through surface contact.[1] When people contact surfaces that have been contaminated with viruses and bacteria, their facial mucosa can become infected consequently. Many studies have found that surface contamination is a strong contributor to the spread of nosocomial infections.[2,3] Conventional methods for disinfecting surfaces employ alcohol solutions, oxidants such as hydrogen peroxide and sodium hypochlorite, and quaternary ammonium surfactants.[4] Such methods, however, are effective only at the time of application as the agents quickly evaporate or are washed or wiped away easily, making the surfaces susceptible for re-contamination. Contaminated surfaces arouse severe danger to patients in healthcare facilities as surface contamination increases the risk of gaining fatal infections such as sepsis. WHO estimates

that 20% of annual global deaths (11 million deaths) are due to sepsis where 25% of which are attained in the hospital, and 33% die as a result.[5] Thus, new disinfecting methods to overcome the drawbacks of conventional disinfectants are in critical need.

Researchers have sought to create "self-disinfecting" surfaces that deliberately inactivate and prohibit pathogens.[6] Current approaches include (1) depositing heavy metal ions such as Ag, Cu, and Zn and (2) incorporating quaternary ammonium compounds. For surfaces with heavy metal ions, disinfection is accomplished through gradual diffusion of soluble metal ions into the pathogen to disrupt their metabolism. This approach, however, depends on gradual diffusion of small amounts of biocide that may take 24 hours to reach effective inhibitory concentration.[7,8] If, by chance, plentiful amounts of metal ions are discharged at once, it may still take at least 5 minutes to initiate inactivation.[9] Thus, this method is not powerful enough in heavy traffic areas such as public transportation hubs as surfaces are constantly contaminated. The rate of recontamination may exceed the rate of ion release to achieve effective concentration.

On the other hand, quaternary ammonium-based coatings accomplish antimicrobial effects through direct contact similar to common cationic detergents. Here, cationic nitrogen components of

the disinfectant bind with anionic lipid components of bacterial membranes viral envelopes to provoke lysis and inactivation of pathogens.[10] Since this method depends upon direct contact with the pathogen and not upon gradual diffusion of a biocide, drawbacks of metal ion-releasing approach are reduced. The ability for quaternary ammonium organosilicon compounds to modify surface with antimicrobial properties was first introduced in 1969 by researchers at Dow.[11] Since then, incorporation of quaternary ammoniums into surface coatings has been an active area of research and led to production of octadecyldimethyl(3-trimethoxysilylpropyl)ammonium chloride, a popular compound used for antimicrobial disinfectants.[12-14] Although this approach in principle can solve primary problems with other self-disinfecting surfaces, it also has drawbacks: 1) Approaches that involve coatings often rely on specialized processes in hazardous solvents or utilize specific techniques that are custom tailored to a particular surface and therefore not sufficiently safe, user-friendly, or widely compatible with the wide variety of surfaces; and 3) Compared to conventional disinfectants, their efficacy is not high enough or the spectrum of efficacy is too narrow.

1.2. Purpose of Research

Considering the drawbacks of biocide-releasing agents and conventional quaternary ammonium silanes, adjustments can be made to the latter. Conventional quaternary ammonium silanes are monoionic surfactants with a single quaternary ammonium group and a hydrophobic tail whereas gemini surfactants have multiple hydrophobic tails and two or more hydrophilic head groups separated by a spacer. The silane bases at the tip of the hydrophilic head group act as anchors to corresponding substrates as they form Si-O bonds with the hydroxyl groups at the surface. The positively charged nitrogen of the hydrophilic head group mechanically draws negatively charged microbial membranes. Meanwhile, the hydrophobic tail of carbons punctures the cell membrane. As the gemini diquaternary organosilane molecule has two hydrophilic head groups and two hydrophobic tails, the possibility of improving the performance of antimicrobial activity can be hypothesized.[15]

Previous studies have found that the carbon chain length of the hydrophobic tail affect antimicrobial efficacy. The effective number or carbons fall between 4 and 18 and the maximum efficacy can be performed with n = 12-18. It is mostly demonstrated that the antimicrobial performance is best exhibited with chain length of n=12-14 for gram-positive and n=14-16 for gram-negative

bacteria.[16] Gram−positive bacteria are characterized by thick, semi−permeable peptidoglycan layer with negatively charged teichoic acids. Underneath this layer is the phospholipid bilayer targeted for penetration. Gram−negative bacteria have another bilayer of phospholipid at their outermost surface, along which negatively charged lipopolysaccharides exist. The peptidoglycan layer of gram−negative bacteria is thinner than that of gram−positive bacteria. Specific composition varies among bacteria and such diversity is assumed to be inducing differing effective carbon chain length for each bacterium.

Thus, three new gemini diquaternary organosilanes with differing alkyl tail lengths of n = 14, 16, and 18 were synthesized to compare their efficacy to those of conventional disinfectants against *S. aureus, E. coli, P. aeruginosa,* and SARS−CoV−2. As SARS−CoV−2 is an enveloped virus with slight negatively charged lipid membrane, it was conjectured that gemini diquaternary organosilanes will be effective against the virus as well. It was assumed that the improvements of newly synthesized disinfectants may stem from more tridentate Si−O bonds formed along the surface with more positively charged nitrogens to attract microbes and penetrate their membranes with more alkyl chains.

2. Materials and Methods

2.1. Preparation of conventional disinfectants

As positive control, two conventional self-sanitizing solutions of mono quaternary ammonium organosilane (octadecyldimethyl(3-trihydroxyoxysilylpropyl)ammonium chloride): MQA and MQB) were employed. MQA was prepared by diluting a 5 w/v% aqueous solution of the pre-hydrolyzed trihydroxysilane octadecyldimethyl(3-trihydroxyoxysilylpropyl) ammonium chloride (HM4005, Gelest, PA, USA). MQB was prepared by hydrolyzing trimethoxysilane octadecyldimethyl(3-trimethoxysilylpropyl) ammonium chloride 72 w/v% in methanol (MeOH). MQ solutions were diluted to 0.1 w/v% with distilled water (DW) that had been adjusted to pH 2 with HCl. Common disinfecting/sanitizing solutions were also employed as additional controls - 70 w/v% ethanol (EtOH), 3 w/v% hydrogen peroxide (H_2O_2), and 0.07 w/v% sodium hypochlorite (NaOCl).

2.2. Synthesis of GQ compounds

Due to limitations of current laboratory setting, chemical synthesis was conducted at University of California, Santa Barbara Marine Science Institute. All reactions were conducted under

anhydrous conditions. Acetonitrile (MeCN) was purchased pre-dried from Acros Organics (Acroseal, NJ, USA) and Et_2O and EtOAc were purchased from Fisher and dried for 72h over 3Å sieves before use. Pre-dried acetone and anhydrous pre-distilled N,N,N'N'-Tetramethyl-ethylene diamine (TMEDA) were purchased from Acros Organics (NJ, USA) and the latter was stored over potassium hydroxide (KOH) pellets. Alkyl chlorides were purchased from TCI (OR, USA).

First step for the synthesis of GQ compounds began with synthesis of 2-(dimethylamino)ethyl-N',N'-dimethylhexadecan-1-amminium chloride. A heavy walled 250ml schlenk flask was first flame dried for 1 minute, sealed with rubber septum, and let cool for 10 minutes. Then, 11 ml of TMEDA was added with syringe, followed by 37 ml of MeCN, and 10 ml of 1-chlorohexadecane. The septum over the valve was replaced with a schlenk valve to seal tightly. The vessel was then placed in a pre-heated oil bath at 75-80℃ and stirred for 6 days. The vessel was then let cool at room temperature and the mixture was moved into a 250ml round bottom flask. Solvent was removed from the mixture in water bath at 35℃ and further evaporated with Et_2O until no scent of TMEDA could be detected. The crude compound was further purified by recrystallization from anhydrous ethyl acetate (EtOAc). After

centrifugation at 1300rpm for 10 minutes, supernatant was removed, and remaining crystals was resuspended in Et_2O. The pellet was quickly transferred to a dry round bottom flask, and the remaining volatiles were removed under high vacuum. The resulting pure product of white powder, 2-(dimethylamino)ethyl-N',N'-dimethylhexadecan-1-amminium chloride, was protected from moisture in tightly sealed vials wrapped with parafilm.

Then, heavy walled schlenk vessel was dried and charged with 2.56 g of the resulting white powder above, 3.76 ml of 3-chloro-1-(trimethoxy)silylpropane, and 6 ml of MeCN. The flask was tightly sealed with a schlenk valve and placed in an oil bath at 120℃, and stirred gently for 72 hours. The vessel was allowed to cool at room temperature. After the schlenk valve was replaced with a rubber septum, anhydrous Et_2O was added with syringe to precipitate the product. The mixture was agitated for several minutes and let stand to remove settled supernatant with a dry syringe and needle. This process was repeated once more with Et_2O, twice with anhydrous acetone, and then a final time with Et_2O. The product was put in dry acetone and transferred to a dry round bottom flask to remove volatiles under reduced pressure, and further dried under high vacuum to obtain the desired N^1-hexadecyl-N^1,N^1,N^2,N^2-tetramethyl-N^2-(3-(trimethoxysilyl)

propyl)ethane-1,2-diaminium dichloride salt as a white hygroscopic powder. The resulting powder was stored in vials tightly sealed with parafilm and named GQ16.

GQ14 was synthesized as described above while using 1-chlorotetradecane instead of 1-chlorohexadecane in step 1. GQ18 was synthesized using 1-chlorooctadecane instead of 1-chlorohexadecane with the following modifications: i) An additional 5-10 vol% of EtOAc was added at step 1 to help solubilize the alkyl chloride; ii) After removal of the volatiles, the intermediate was triturated with cold Et_2O, and then recrystallized from hot MeCN; iii) Following the step above, additional anhydrous MeCN was added to precipitate. Colored impurities were removed and the crude material was recrystallized from boiling MeCN and triturated several times with anhydrous Et_2O until white powder was obtained. We evaluated these new compounds as dilute (ca. 0.1%w) solutions.

2.3. Preparation of pathogens

Pathogens tested in this study were common nosocomial bacteria: *S. aureus* (ATCC 29213), *E. coli* (ATCC 67024), and *P. aeruginosa* (ATCC 27853). *S. aureus* and *E. coli* were provided by Korea Collection for Oral Microorganisms (KCOM) and *P. aeruginosa* (ATCC 27853) was provided by the American Type

Culture Collection, Manassas, VA. SARS-CoV-2 was provided by SGS in Geneva, Switzerland. The media used for the culture of *S. aureus* was tryptic soy (TS, MB cell) and Luria Bertani (LB, MB cell) for *E. coli* and *P. aeruginosa*. A sample of each bacterium in fresh broth medium was prepared and spread over the corresponding agar plate to be incubated for 24 hours. A colony was transferred to phosphate buffered saline (PBS) and vortexed. Coronavirus was grown for 2-7 days at 37° C in MRC-5 cells at 5% CO_2 atmosphere. Each suspension was diluted by a factor of ten and spectrophotometrically adjusted to a final concentration of 1.0 x 10^6 colony-forming units (CFU) or plaque-forming units (PFU) per mL of suspension.

2.4. Suspension antimicrobial efficacy test

Antimicrobial efficacy in suspension test was performed based on ASTM E2315 (Suspension time-to-kill test for bacteria). Test for each solution was repeated 5 times (n=5). A vacuum filtration apparatus with membrane filter (Millipore membrane filter, 0.45μm pore size) was used, into which 5ml of testing solutions with 100 microliters of bacterial suspension was decanted and set for 15 seconds. The vacuum was released and sufficient amount of D/E neutralizing buffer was poured. The membrane filter was

transferred onto the agar plate and 100μl of PBS was dropped to spread evenly with glass cell spreader. The plates were incubated for 24 hours at 37℃ to count for the number of surviving colonies.

SARS-CoV-2 viral test was conducted at a contract research organization, TransPharm (MI, USA) that satisfies the biosafety level requirements (BSL-3). The viral suspension was tested following the adequate test standard ASTM E1052 (Suspension time-to-kill test for virus). Each testing solution was dispensed to a 96-well plate. Then, viral suspension was added to each well and held for 15 seconds before neutralized by ten-fold serial dilutions into the appropriate solution. Following neutralization, the viral suspension was tested using RT-PCR test kit (ViroReal Kit, Ingenetix GmbH, Wien, Austria). The cycle threshold (CT) values, the number of cycles necessary to spot the virus, were generated via RT-PCT test as viral load indicators.[17-19]

2.5. Antimicrobial efficacy on non-porous surface

Antimicrobial activity on non-porous surface was tested based on ISO 22196 (Antibacterial activity on non-porous surfaces). Test for each solution was repeated 5 times (n=5). Sterilized 75×25 mm glass sheets were prepared, onto which testing solutions were spread evenly. The surfaces were air-dried

with lens blower until no trace of solutions could be seen with bare eyes. The air-drying times were 5 minutes, 1 hour, and 24 hours. For GQ18 and GQ16, we allowed additional air-drying time of 15 days for test of long-term efficacy. Then, 100ul of bacterial suspension was pipetted on the surface to be covered with 65×15 mm sterile polyethylene film. The glass plate was placed on a 60×60×10mm plastic mesh rack, together which was placed in a sterile 90mm petri dish with 1 ml of DW to prevent dehydration. The specimens were incubated for 24 hours at 37 ℃. The specimens were then put into a Stomacher bag of 5ml PBS to be further put into ultrasonic cleaner for 5 minutes. Tenfold serial dilution was made with PBS, and 100μl of each dilution was spread on agar plates. The plates were incubated for 24 hours at 37℃. The number of surviving colonies was determined with the following equation:

$$N = (100 \times C \times D \times V) / A$$

where C is the average CFU count, D is the dilution factor, V is the volume of PBS added to the Stomacher bag, and A is the surface area of the cover film.

SARS-CoV-2 viral test was conducted at a contract research organization, TransPharm (MI, USA) that satisfies the biosafety level requirements (BSL-3). Since there is no international guideline specified for the contact transmission test

against SARS-CoV-2, modified ISO 21702 and ISO 18184 protocols were followed. An RT-PCR test for 0.08% GQ18 against SARS-CoV-2 was conducted on a stainless-steel cylinder to simulate a doorknob. The cylinder was contaminated by swabbing with a wet cotton ball contaminated with a high viral load (CT<25). Then, GQ18 solution was sprayed thoroughly over the cylinder. After 15 seconds, the surface was swabbed for RT-PCR test using ViroReal Kit (Ingenetix GmbH, Wien, Austria). The cycle threshold (CT) values, the number of cycles necessary to spot the virus, were generated via RT-PCT test as viral load indicators.[18-20]

2.6. Antimicrobial efficacy on porous surface

Antimicrobial activity test on porous surface was tested based on ISO 20743 (Antibacterial activity on porous surfaces). Test for each solution was repeated 5 times (n=5). Sterilized 50× 25mm (0.4g) neoprene fabric specimens were wet both sides with 200μl of test solution. The fabric pieces were air-dried for 5 minutes, 1 hour, and 24 hours. The specimens were then put into conical test tubes with 5ml of culture broth and PBS mixture and 100μl of prepared bacterial suspension. After incubation for 24 hours, the specimens were vortexed for 1 minute. The specimens were then sampled following the same procedure described above.

2.7. Contact angle measurement

Sterilized 75× 25 mm glass sheets were prepared to study the surface energy of quaternary ammonium-based antimicrobial solutions. The drops of prepared solutions were measured for contact angles on the plane glass slides. The sterilized glass slides were sprayed with the MQ and GQ solutions followed by air-drying to measure the DW drop contact angles. Contact angles were measured 1 hour and 24 hours after the air-drying was complete and averaged (n=5). Images were taken with a digital single-lens reflex camera (Nikon) and analyzed with ImageJ.

2.8. Mechanical durability on non-porous surface

The weight pressing abrasion method modified from ISO 10993 (Biological evaluation of medical devices) was used to test mechanical durability on non-porous surfaces.[20] The test solutions were sprayed and spread evenly on sterilized 75 × 25 mm glass sheets. The glass sheets were air-dried with a lens blower and further dried in ambient condition for 24 hours. Then, the surface was swabbed with dry and wet cotton swabs under a pressure of 8.8 kPa for 10 times using universal testing machine (TW-D102, Taewontech, Korea). After the dry and wet abrasion was applied, DW was dropped on the glass sheets to measure contact angles

(n=5). Then, the remaining surfaces were inoculated with *S. aureus* and *E. coli* to examine surviving bacterial colonies following the same procedure described in 2.5. Antibacterial efficacy on non-porous surface (n=5).

2.9. Chemical durability on non-porous surface

Durability of GQ in comparison with MQ to chemical cleansing was tested. GQ18, GQ16, and MQB were each applied to 5 sterilized glass surfaces, air-blown, and let air-dried for one hour in ambient condition. Then, the surfaces were thoroughly rinsed with MeOH and water and analyzed with Thermal-Fisher Nicolate 9700 FTIR fitted with an attenuated total reflectance attachment.

2.10. Residual efficacy on non-porous surface after re-contaminations

Durability of GQ on various non-porous surfaces after repeated contamination was test. MQB, GQ18, and GQ16 were applied evenly on 5 samples of glass, stainless steel, and acrylic plastic slides, air-blown, and let air-dried for 5 minutes in ambient condition (n=5). Subsequently, the surfaces were inoculated with *S. aureus* and *E. coli* and incubated for 24 hours. The surface was re-inoculated every 24 hours for a total of 3 cycles. The surfaces were

then sampled following the same procedure described above in 2.5. Antibacterial efficacy on non-porous surface.

2.11. Cytotoxicity test

Mouse L929 fibroblasts (NCTC clone 929, Korean Cell Line Bank, Seoul, Korea) were cultured in Eagle's minimum essential medium (MEM) supplemented with 10 v/v% fetal bovine serum (FBS), and penicillin-streptomycin antibiotic solution in 5% CO_2 incubator at 37℃.

The L929 cells were seeded and incubated in 96-well plates until confluence. The media was replaced with fresh media containing serial dilutes of GQ18, GQ16, and MQB and incubated for 24 hours. Cell viability was measured with Cell Count Kit-8 (Dojindo Laboratories, Kumamoto, Japan). The OD_{450} was recorded using a Synergy H1 multi-mode microplate reader (Biotek, Winooski, VT, US) and averaged (n=3).

3. Results

3.1. Suspension antimicrobial efficacy

For suspension tests against *S. aureus*, EtOH, H_2O_2, NaOCl, and GQ18 showed ~99.9998% reduction while GQ16 and GQ14 showed decent antibacterial activities of ~99.95% reduction **(Figure 1)**. MQ solutions, however, showed minimal efficacy of 79.6872% for MQA and 90.4243% for MQB.

For suspension tests against *E. coli*, MQA showed minimal efficacy of 76.9978% while EtOH, H_2O_2, NaOCl, MQB, GQ18, GQ16, and GQ14 exhibited ~99.9998% reduction **(Figure 2)**.

P. aeruginosa was inactivated easily with all testing solutions except MQ solutions. While all the other 6 solutions exhibited ~99.9998% reduction, MQA showed 80.5016% and MQB showed 80.9454% reduction **(Figure 3)**.

Furthermore, GQ18 against the coronavirus in suspension showed CT values of 37, 38, and 36 after 15 seconds of contact time **(Figure 5)**.

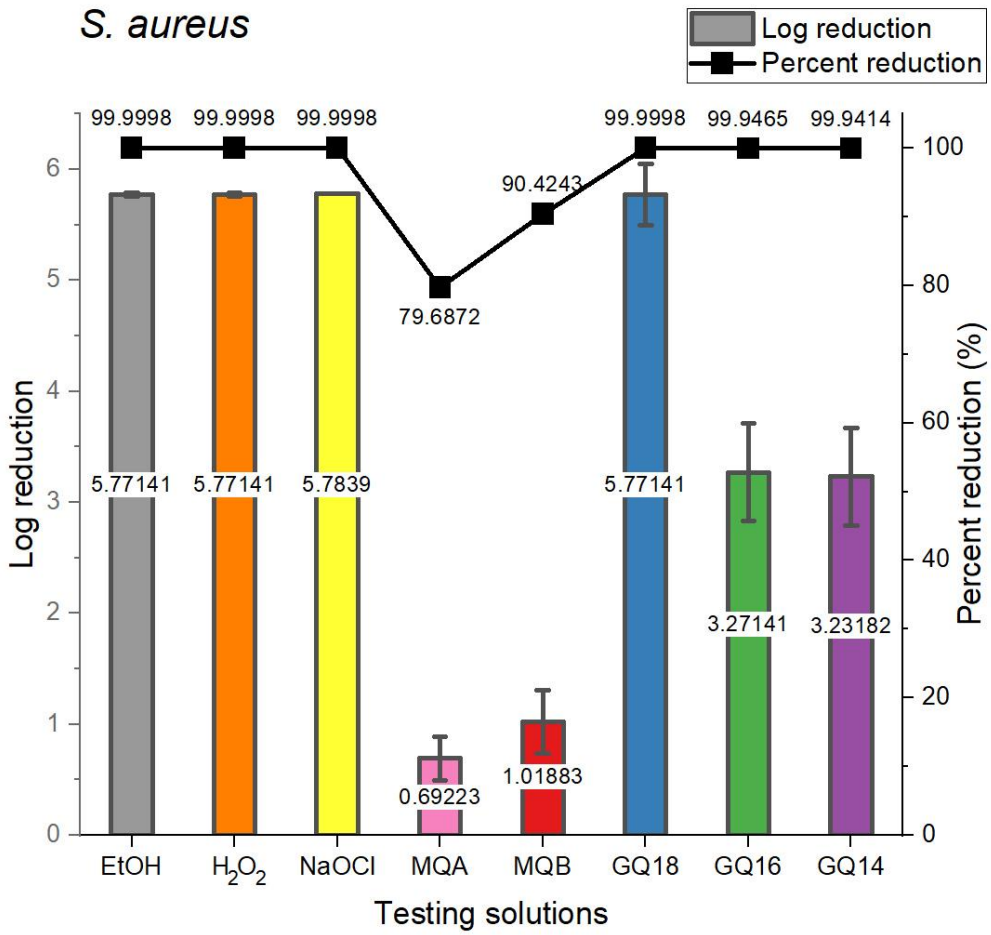


Figure 1. Log and percent reduction measure of antimicrobial solutions at 15 seconds against *S. aureus* in suspension.

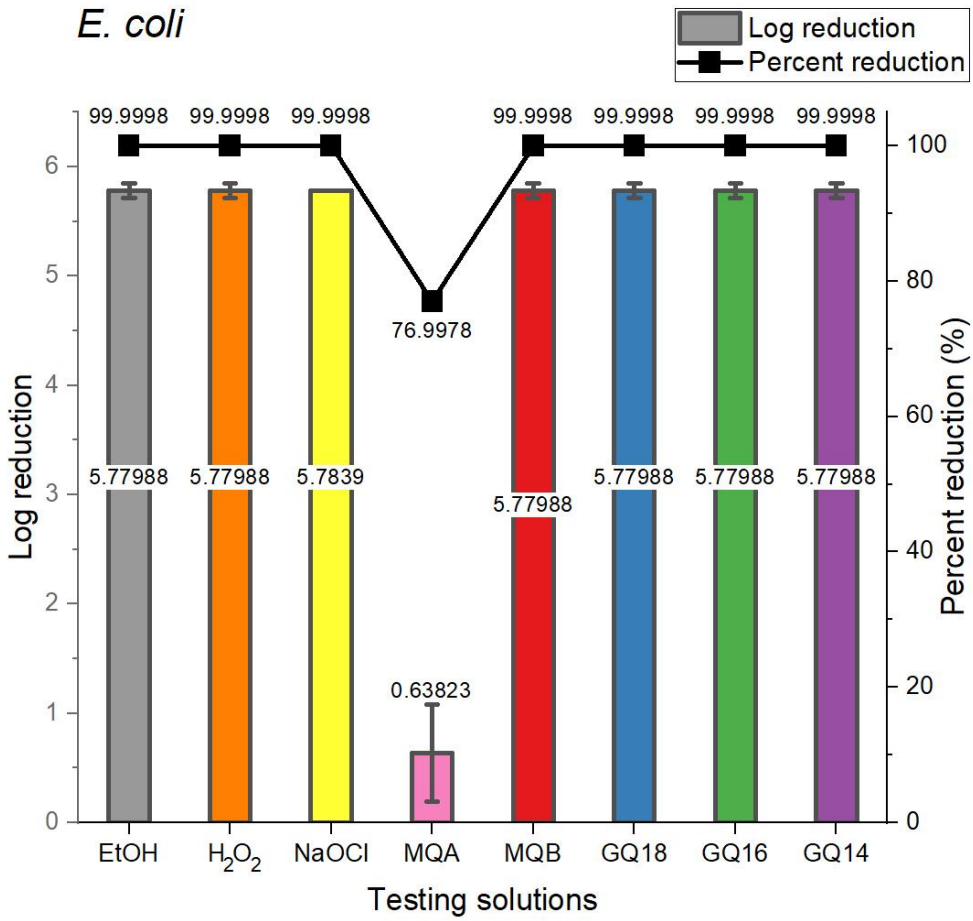


Figure 2. Log and percent reduction measure of antimicrobial solutions at 15 seconds against *E. coli* in suspension.

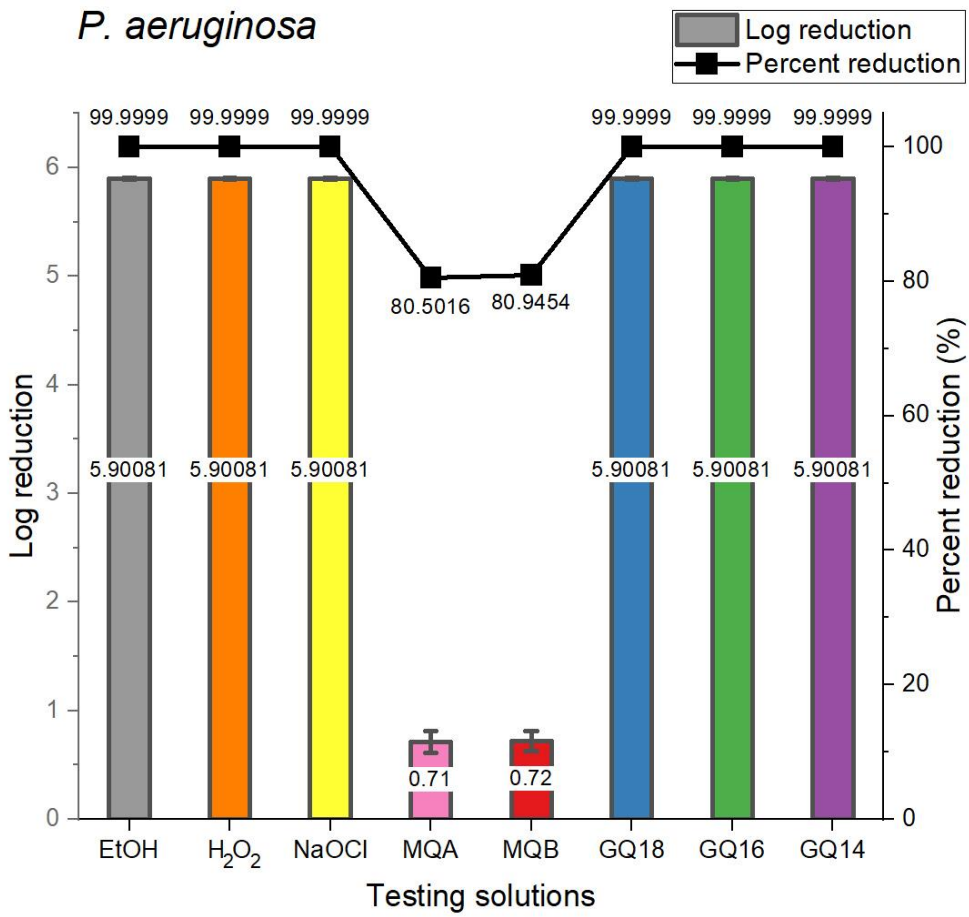


Figure 3. Log and percent reduction measure of antimicrobial solutions at 15 seconds against *P. aeruginosa* in suspension.

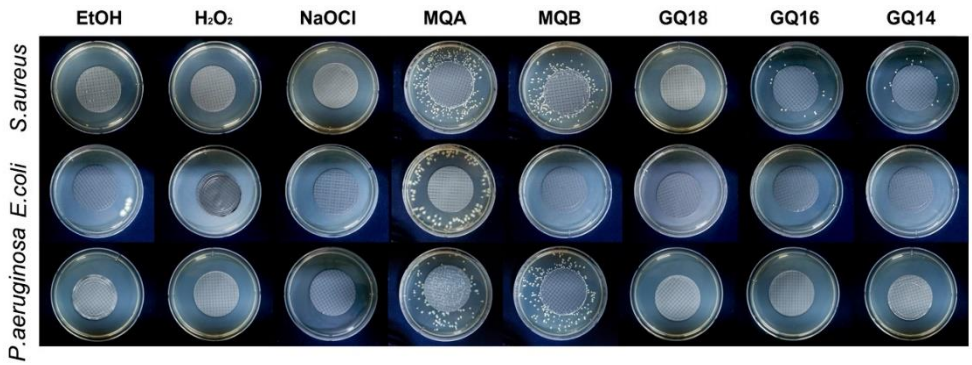


Figure 4. Surviving colony formation of *S. aureus* and *E. coli.*

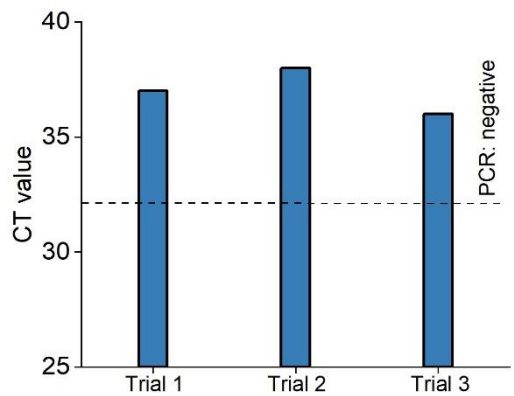


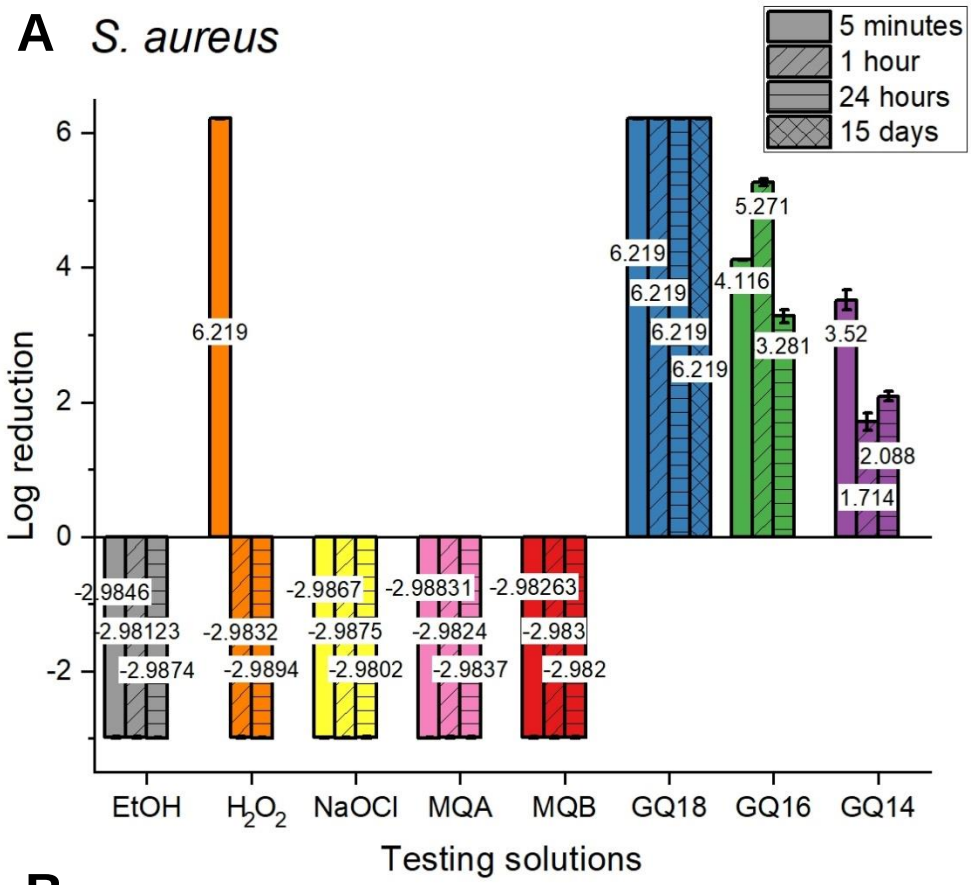
Figure 5. CT values from RT-PCR test for GQ18 against SARS-CoV-2 in suspension at 15 seconds contact time.

3.2. Surface antimicrobial efficacy on non-porous surface

For *S. aureus*, H_2O_2 was effective at 5 minutes, showing 99.9999% reduction **(Figure 6)**. For longer aging times of 1 hour and 24 hours, H_2O_2 lost efficacy against *S. aureus* and colony formation increased by ~95399%. Until 24 hours of aging time, GQ18 maintained the highest efficacy against *S. aureus* while GQ16 and GQ14 showed a trend of decreasing activity. *S. aureus* colony formation was not suppressed by EtOH, NaOCl, MQA, and MQB, but increased by more than ~95000%. At 15 days of aging, GQ18 demonstrated 99.9999% reduction of colony formation.

For *E. coli*, only GQ16 suppressed colony formation by ~99.9998% for all aging times including additional aging time of 15 days **(Figure 7)**. The only other solutions that showed some suppression of bacterial activity were EtOH, H_2O_2, and GQ18 at 5 minutes of aging, which decreased colony formation by 14.1323%, 27.0861, and 41.3146%, respectively. Bacterial activity was increased by ~29340% for NaOCl, MQA, and MQB at all aging times.

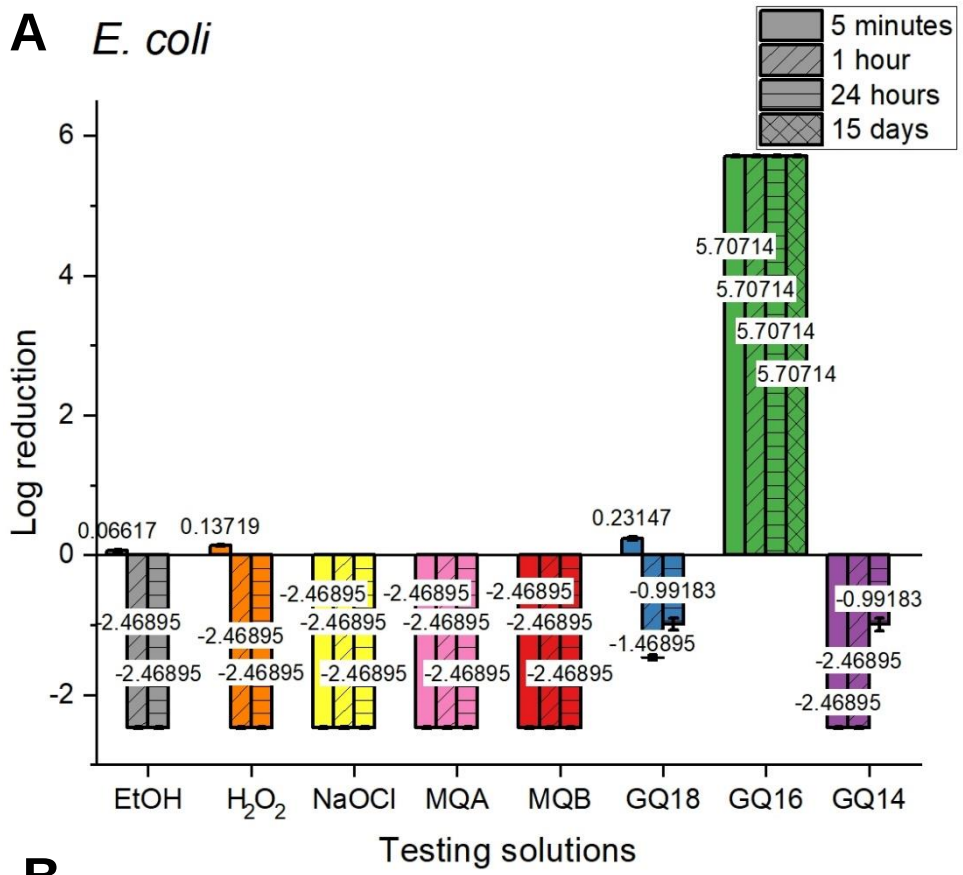
The test results for coronavirus conducted 15 seconds after application of GQ18 showed CT values of 35 **(Figure 9)**. Another RT-PCR test was performed after 6 hours, and the resulting CT value was >45, which is above detectable range. Furthermore, the CT value after 48 hours of contamination and dry was >45.



B

	5 minutes	1 hour	24 hours	15 days
EtOH	-96416.1523	-95670.1130	-97040.4252	
H_2O_2	99.9999	-96105.5219	-97488.8050	
NaOCl	-96883.9794	-97062.7952	-95443.2478	
MQA	-97244.1820	-95928.4679	-96216.3464	
MQB	-95979.3375	-96061.2278	-95840.0632	
GQ18	99.9999	99.9999	99.9999	99.9999
GQ16	99.9923	99.9995	99.9476	
GQ14	99.9698	98.0680	99.1834	

Figure 6. Log reduction (A) and percent reduction (B) measure of antimicrobial solutions coated on non-porous surfaces against *S. aureus* at different aging times.



B

	5 minutes	1 hour	24 hours	15 days
EtOH	14.1323	-29340.9270	-29340.8343	
H_2O_2	27.0862	-29341.1453	-29340.7803	
NaOCl	-29340.9961	-29340.7772	-29340.9893	
MQA	-29340.7202	-29340.7464	-29340.6822	
MQB	-29340.9622	-29340.8946	-29340.6362	
GQ18	41.3146	-2844.0827	-881.3646	
GQ16	99.9998	99.9998	99.9998	99.9998
GQ14	-29340.9012	-29340.7555	-881.3663	

Figure 7. Log reduction (A) and percent reduction (B) measure of antimicrobial solutions coated on non-porous surfaces against *E. coli* at different aging times.

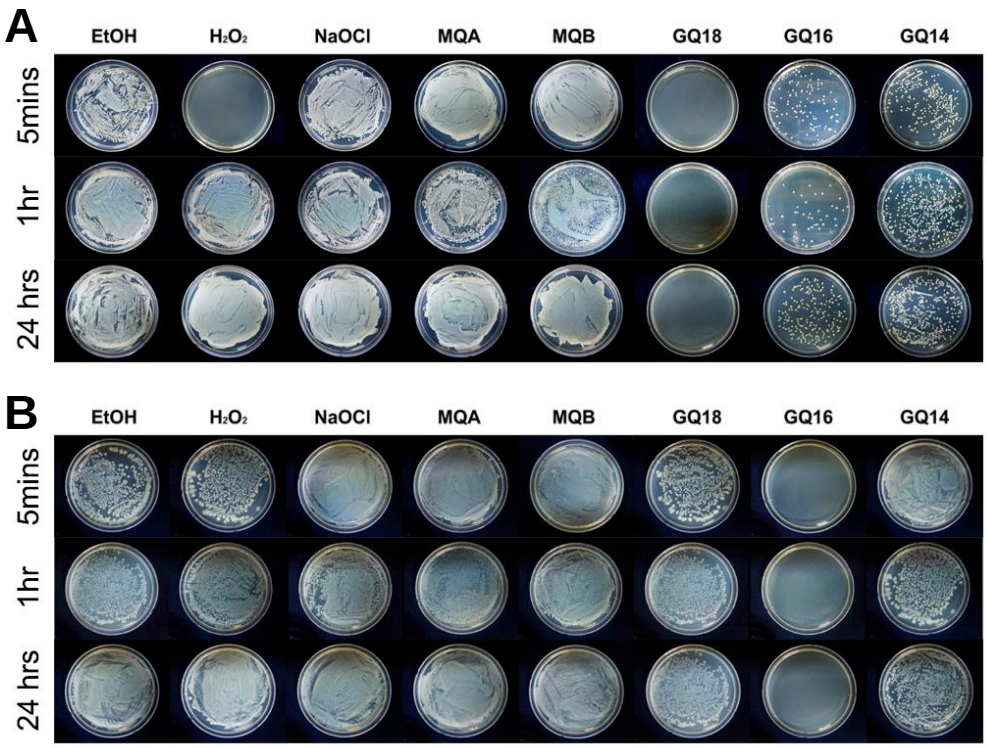


Figure 8. Surviving colony formation of (A) *S. aureus* and (B) *E. coli* after 24 hours of application on non-porous surfaces.

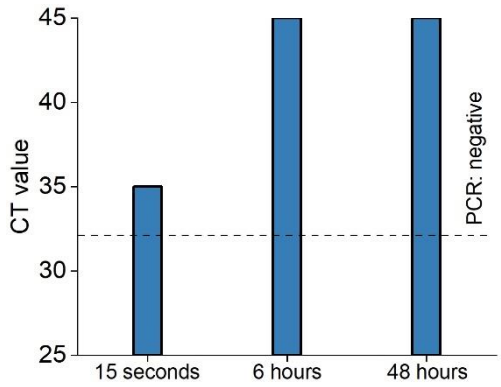


Figure 9. CT values from RT-PCR test for GQ18 coated non-porous surfaces against SARS-CoV-2 at different aging times.

3.3. Surface antimicrobial efficacy on porous surface

Colony formation of *S. aureus* was suppressed completely in H_2O_2, NaOCl, and all GQ solutions for all aging times **(Figure 10)**. EtOH and MQ solutions showed no antimicrobial activities. For these solutions, bacterial colony formation was increased by more than 95000%.

Test against *E. coli* showed H_2O_2, NaOCl, and GQ16 maintaining long-lasting efficacy of 99.9999% until 24 hours of aging **(Figure 11)**. While GQ18 at 5 minutes showed an increase of bacterial activity by 8941%, all the other solutions at all aging times exhibited more than 90314% of increase in colony formation.

3.4. Contact angle measurement

The average contact angle of MQB solution droplets on pristine glass surfaces was larger than those of GQ solutions, forming 50.0±1.3° **(Figure 13)**. GQ18 and GQ16 solution droplets on pristine glass surfaces resulted in average angles of 37.9±2.7° and 41.6±1.0° , respectively.

The average contact angle of DW droplets on MQB coated glass surface after 1 hour was 85.1±2.2944° while those on GQ18 and GQ16 coated glass surfaces were much smaller, forming 50.6± 0.5° and 57.8±2.8° , respectively **(Figure 14)**. After 24 hours, the

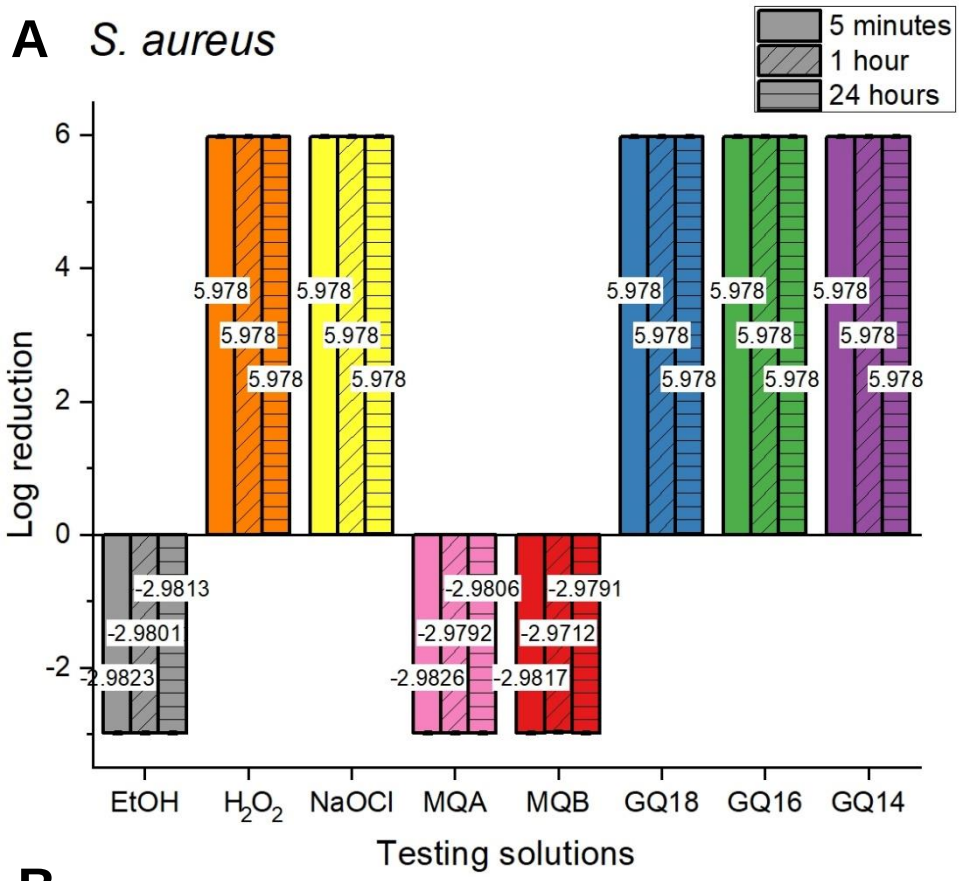
average contact angle of DW droplets on MQB coated glass surface was the largest, forming 77.6±3.2° . The average DW droplet contact angles on GQ18 and GQ16 coated glass surfaces were 67.1 ±1.1° and 69.9±1.0° , respectively.

Additional tests for GQ18 and GQ16 coated glass surfaces were conducted after 15 days **(Figure 15)**. The average DW droplet contact angles on GQ18 and GQ16 surfaces did not increase and remained almost the same at 66.9±2.7° and 67.2±1.4° , respectively.

3.5. Mechanical durability on non-porous surface

The average contact angle of DW droplets on the GQ18 coated glass surfaces after 1 hour was 50.6±0.5° and 57.8±2.8° on GQ16 coated glass surfaces **(Figure 16)**. When mechanically rubbed with dry cotton swabs, the angle increased to 61.1±2.2° and 46.1±2.2° for GQ18 and GQ16, respectively. With wet cotton swabs, the angles increased more dramatically to 73.0±1.4° and 71.3±2.0° , for GQ18 and GQ16 coated surfaces respectively.

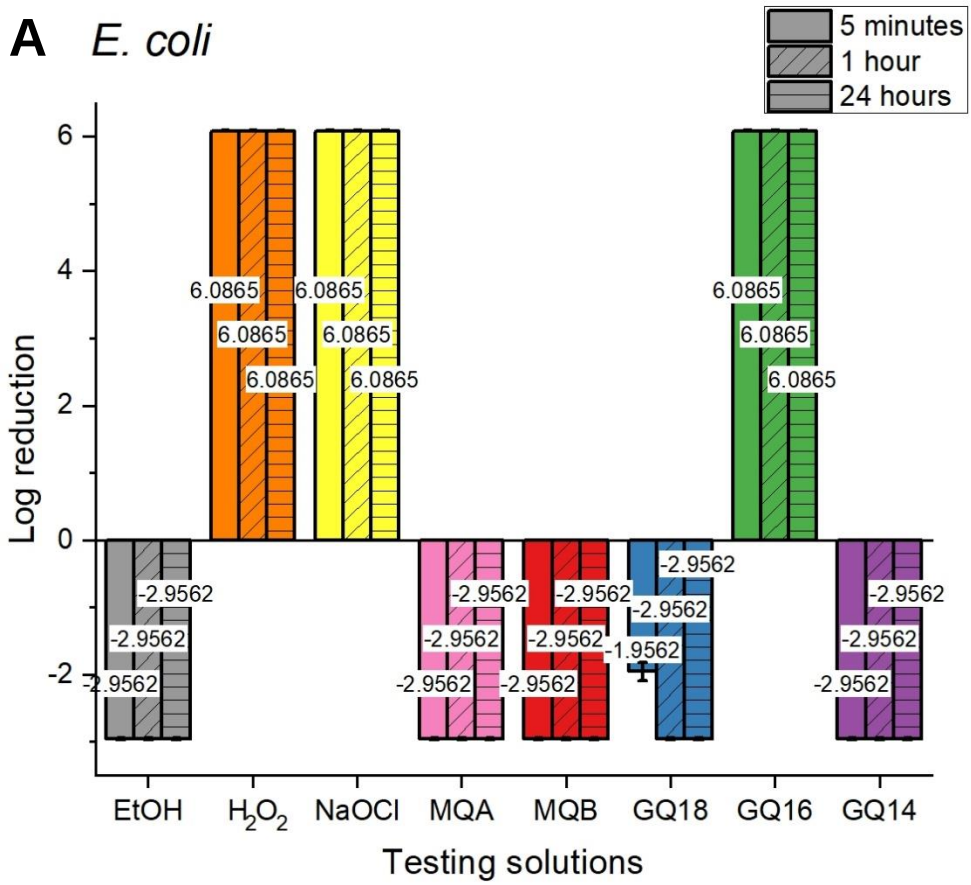
The antimicrobial efficacy test against *S. aureus* and *E. coli* performed on these glass slides after dry and wet swabbing showed maintenance of complete bacterial activity suppression even after application of mechanical abrasion **(Figure 17)**.



B

	5 minutes	1 hour	24 hours
EtOH	-95915.2020	-95430.0489	-95676.7288
H_2O_2	99.9999	99.9999	99.9999
NaOCl	99.9999	99.9999	99.9999
MQA	-95966.7282	-95221.8143	-95520.2776
MQB	-95773.8130	-93490.3777	-95204.8495
GQ18	99.9999	99.9999	99.9999
GQ16	99.9999	99.9999	99.9999
GQ14	99.9999	99.9999	99.9999

Figure 10. Log reduction (A) and percent reduction (B) measure of antimicrobial solutions coated on porous surfaces against *S. aureus* at different aging times.



B

	5 minutes	1 hour	24 hours
EtOH	-90314.6732	-90315.3546	-90314.9466
H_2O_2	99.9999	99.9999	99.9999
NaOCl	99.9999	99.9999	99.9999
MQA	-90314.9258	-90315.1152	-90314.6605
MQB	-90315.5857	-90315.0519	-90315.3588
GQ18	-8941.4899	-90315.0049	-90315.1110
GQ16	99.9999	99.9999	99.9999
GQ14	-90314.6815	-90314.8779	-90314.9049

Figure 11. Log reduction (A) and percent reduction (B) measure of antimicrobial solutions coated on porous surfaces against *E. coli* at different aging times.

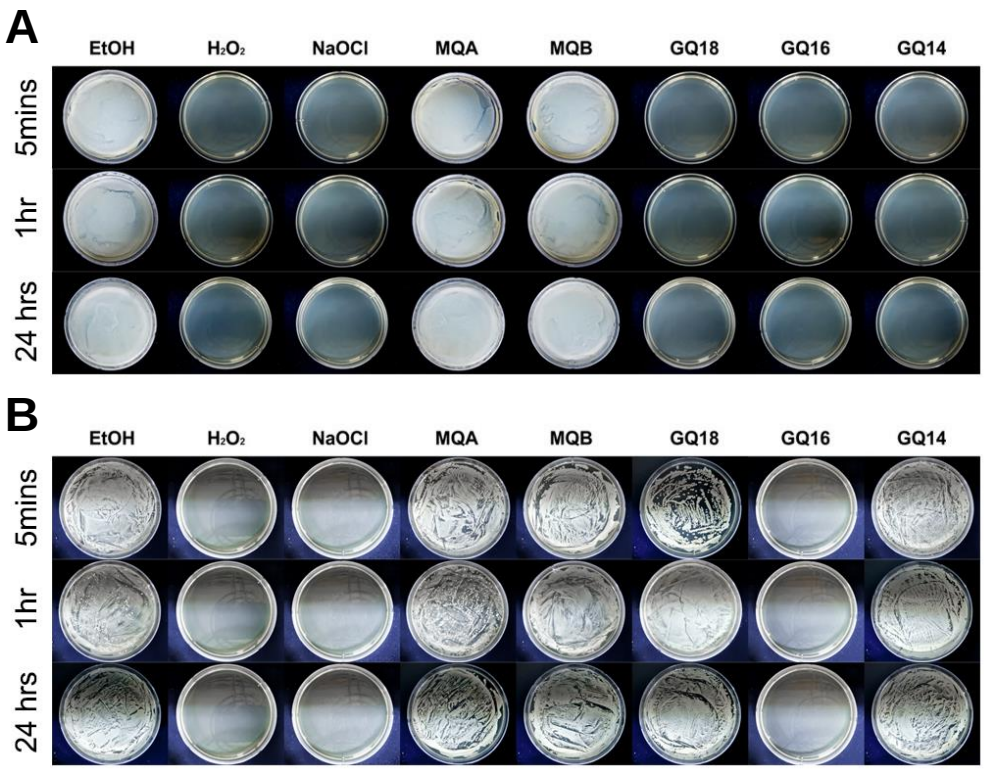


Figure 12. Surviving colony formation of **(A)** *S. aureus* and **(B)** *E. coli* after 24 hours of application on porous surfaces.

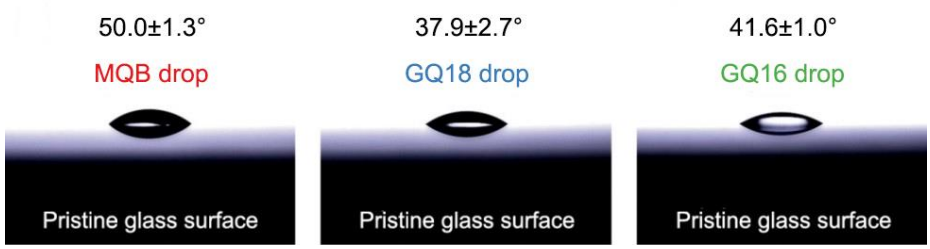


Figure 13. Contact angle measurements of droplets of solutions on pristine glass slides.

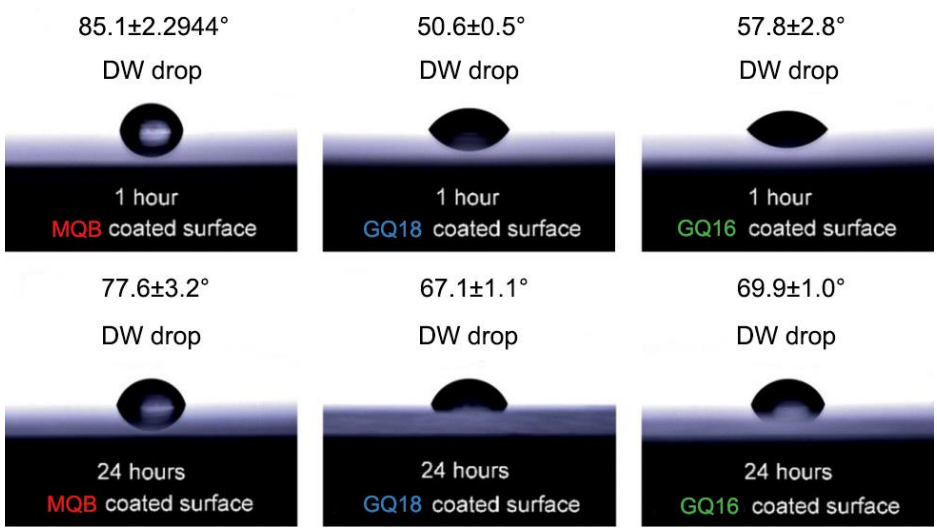


Figure 14. Contact angle measurements of DW droplets on solution coated glass slides after 1 hour and 24 hours of aging.

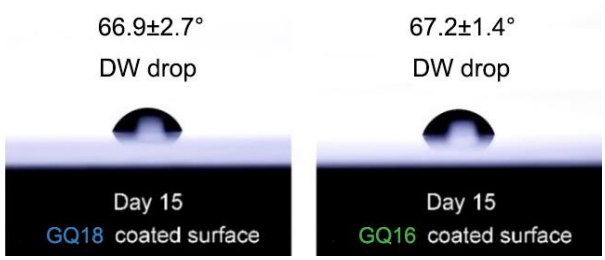


Figure 15. Contact angle measurements of DW droplets on solution coated glass slides after 15 days of aging.

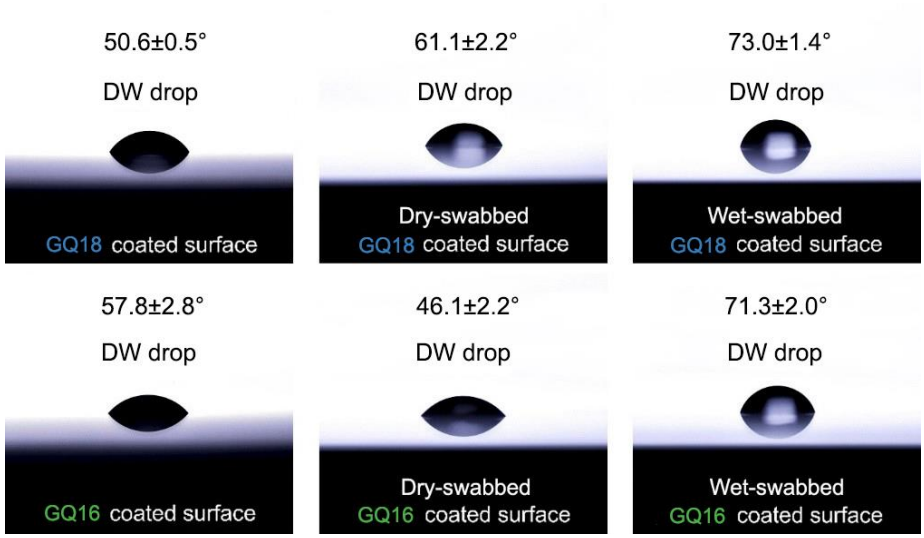


Figure 16. Contact angle measurements of DW droplets on solution coated glass slides after 1 hour.

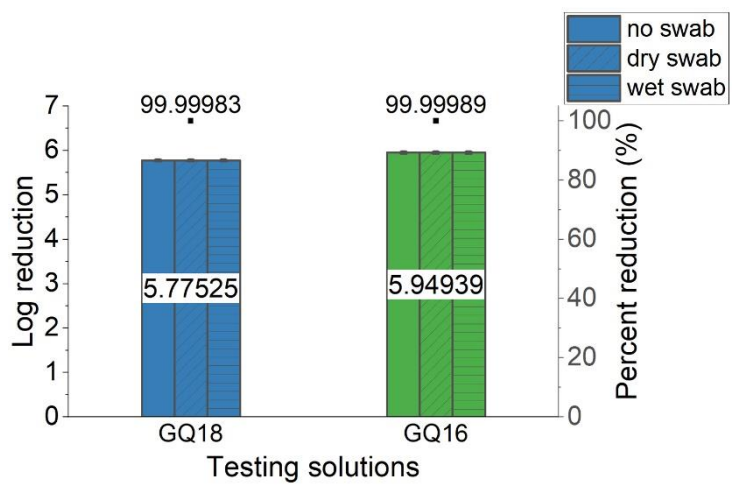


Figure 17. Log reduction and percent reduction measure of antimicrobial solutions coated on non-porous surfaces.

3.6. Chemical durability on non-porous surface

FTIR spectra of GQ coated slides that were not rinsed indicated a higher degree of deposition compared to the conventional MQ solution that showed almost no alkyl C-H stretch (Figure 18). When rinsed, the C-H stretch of GQ18 and GQ16 coated slides exhibited decreased deposition.

3.7. Residual efficacy on non-porous surface after re-contaminations

The efficacy of both GQ18 and GQ16 was retained on all three surfaces, even after three repeated contaminations (Figure 19). On the contrary, MQB showed no efficacy on any of the surfaces against both *S. aureus* and *E. coli*. GQ18 coated glass maintained 99.9999% of bacterial activity reduction while GQ18 coated stainless steel and plastic presented average ~99.99% reduction against *S. aureus*. GQ16 coated glass showed 9.9999% reduction. Furthermore, GQ16 coated stainless steel and plastic demonstrated ~99.999% reduction.

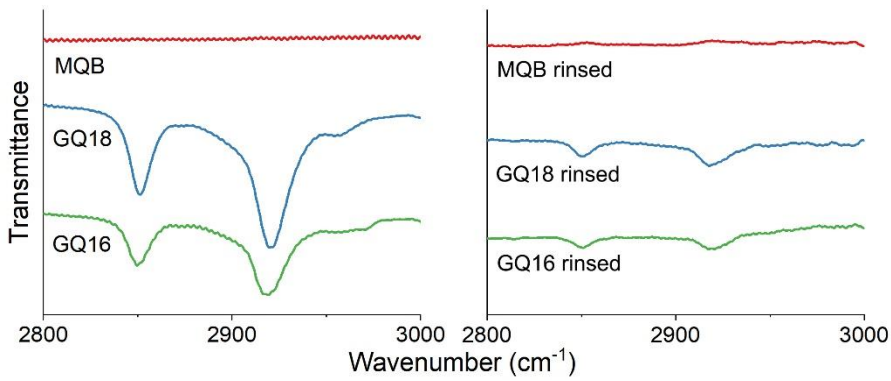
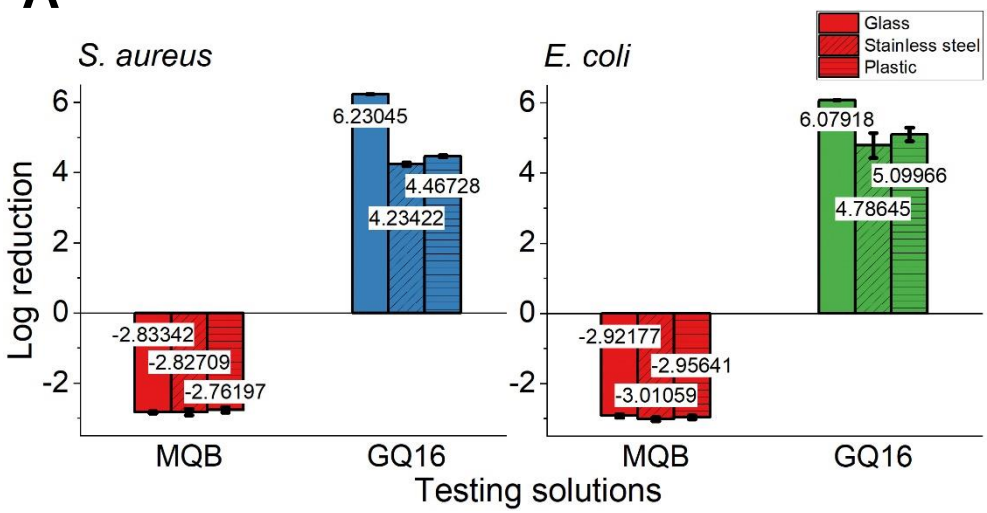


Figure 18. FTIR spectra of pristine glass coated with MQB, GQ18, and GQ16 without and with rinsing.

A



B

	S. aureus		*E. coli*	
	MQB	**GQ18**	**MQB**	**GQ16**
Glass	-68042.8039	99.9999	-83416.0605	99.9999
Stainless steel	-67056.8009	99.9942	-102368.410	99.9984
Plastic	-57705.6115	99.9966	-90350.2976	99.9992

C

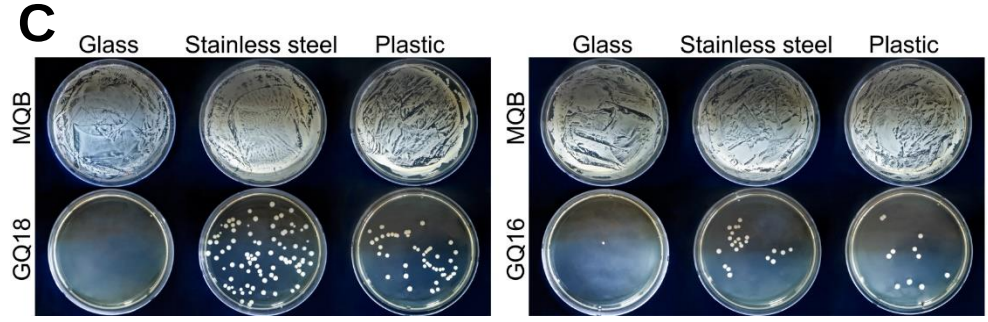


Figure 19. Log reduction (A) and percent reduction (B) measure of residual antimicrobial activities on three different types of non-porous surfaces against *S. aureus* and *E. coli*; (C) Surviving colony formation *S. aureus* and *E. coli* on three different non-porous surfaces.

3.8. Biocompatibility

MQB was moderately toxic with an LC_{50} at 0.07504 wt% (Figure 20). The LC_{50} value of GQ18 was 0.08871 wt%, slightly more dilute than the concentration (0.1 wt%) used to generate effective coatings in all the tests. GQ16 showed the highest biocompatibility with LC_{50} occurring at 0.1457 wt%. At 0.1 wt%, MQB showed lowest cell viability of 21.72%l while GQ18 and GQ16 presented 27.53% and 74.66% cell viability.

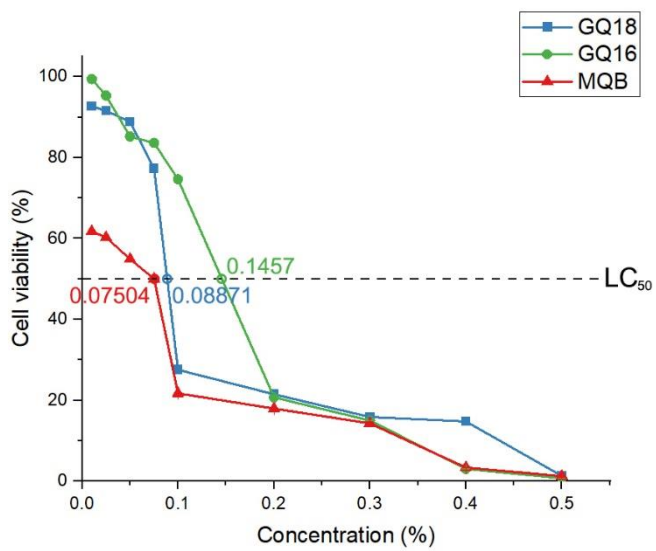


Figure 20. Cell viability and LC_{50} values of MQB, GQ18, and GQ16.

4. Discussion

To determine the best performing gemini diquaternary organosilanes for surface antimicrobial activity, evaluations of antibacterial and virucidal activity were conducted based on ASTM E2315 (suspension time-kill test for bacteria) and ASTM E1052 (suspension time-kill test for virus). Newly synthesized gemini diquaternary (GQ) and conventional monoquaternary (MQ) silanes were evaluated as 0.1% aqueous solutions, while common disinfectants were used as controls: 70% ethanol (EtOH), 3% hydrogen peroxide (H_2O_2), and 0.07% sodium hypochlorite (NaOCl). The antimicrobial activity of disinfectants was tested against common gram-positive bacteria *S. aureus* and negative bacteria *E. coli* and *P. aeruginosa* using a mean initial inoculum of 1.0×10^6 CFU/ml for each test. The contact time was set at 15 seconds because increasing the time in suspension leads to indistinguishable results.

For both *S. aureus* and *P. aeruginosa*, antibacterial efficacy of MQ solutions was relatively minimal compared to those of the other solutions. For *E. coli*, only MQA exhibited lower antibacterial efficacy. These suggest that newly synthesized GQ solutions, which comprise monomers with additional hydrophilic head and

hydrophobic tail group, better attract negatively charged membranes of bacteria and puncture their membrane more efficiently.

Meanwhile, the diminished performance of MQA relative to MQB can be associated with the inverse relationship between stability and concentration of aqueous solutions of hydroxysilanes.[21] It is known that alkoxysilanes in anhydrous alcohol are stable against hydrolysis in behalf of excess alcohol. Dilute aqueous solutions (<0.5%) of trihydroxysilanes are also stable, but the more concentrated, the more prone they are to gradual self-oligomerization. This is because the rate of silanol condensation to siloxanes is a second order reaction in silanol, and is exponentially faster with increasing concentration.[19] The 0.1% MQA prepared from a 5% aqueous solution of trihydoxysilane may be substantially more oligomerized before dilution than 0.1% MQB, which was diluted and hydrolyzed to 0.1% from a stable trimethoxysilane.

Along with common nosocomial bacteria, the efficacy of GQ solutions against coronavirus was evaluated as well. Only one sample (0.1% GQ18) was tested due to limited resources. RT-PCR test was conducted to provide CT value. A CT value of <25 is generally considered a high viral load with a high risk of transmission, whereas a value of >30 is considered a low risk of

transmission. A value >32 is considered no risk of transmission.[17] The resulting values of 37, 38, and 36 indicate that GQ18 effectively suppressed viral activity, and thus GQ18 can be used as virucidal agent to inactivate SARS-CoV-2.

For the test on non-porous surfaces, GQ solutions were effective against *S. aureus* at all aging times. The only non-quaternary ammonium silane based disinfectant that showed antibacterial activity against S. aureus on non-porous surface was H_2O_2 at 5 minutes of aging. For the test against *E. coli*, only GQ16 suppressed bacterial growth sufficiently. Complete loss of antibacterial activity of EtOH, H_2O_2, and NaOCl is assumed to be due to their volatility. EtOH evaporates, H_2O_2 decomposes to water and O_2, and NaOCl degrades to chloride and O_2 at room temperature. Such volatility was accelerated by blow-drying process.

It was surprising to find that conventional monoionic organosilane MQA and MQB have no long-term surface germicidal efficacy because they had been claimed as popular antimicrobial coating agents. Presumably, their poor efficacy may be due to their lower surface energy that decrease ability to spread, making them easy to and self-assemble upon the surface and be removed by air-blowing.

For the test against virus, CT values greatly exceeded 32,

the marginal value for negative target viral DNA fragment detection. Such result suggests that GQ18 has great potential to reduce the surface-transmission of SARS-CoV-2 and possibly other enveloped viruses.

The antimicrobial efficacy of the solutions on porous surfaces (neoprene fabric) against *S. aureus* and *E. coli* was tested with spraying, blow-drying, and subsequent aging. Efficacy of conventional disinfectants H_2O_2 and NaOCl until 24 hours is suggestive of the absorption and release mechanism within the fabric, absorbing and releasing biocides over and over again. However, H_2O_2 and NaOCl are not suitable for the use on dermal tissue because they are chemically cytotoxic.

GQ18 and GQ16 show complementary activity against gram-positive and gram-negative bacteria, respectively. While both GQ18 and GQ16 were effective against gram-positive *S. aureus*, only GQ-16 showed superior efficacy against gram-negative *E. coli*. This difference is likely due to gram-specific variations in membrane compositions of the bacteria and corresponding ability of the alkyl tails of 18- and 16- carbon homologs to intercalate within the membrane.[10,22] This result is consistent with prior studies: 1) similar efficacy observed against gram-positive strains for surfactants with 16- and 18-carbon *n*-alkyl chains, and 2) highest

efficacy against gram-negative strains for compounds posessing a 16-carbon alkyl tail.[10,23]

Different gram-specific efficacy of GQ18 and GQ16 against gram-positive and gram-negative bacteria was more prominent in the surface antimicrobial tests compared to suspension test. This may be explained by the differences in the mechanism of action for static-antimicrobial coatings and water soluble disinfectants. Soluble cationic disinfectants exhibit maximum efficacy near their critical-micelle-concentration, as there is a sufficiently high amount of monomers available to diffuse into bacterial membranes to trigger cell lysis.[22] In suspension, disinfectant monomers can swiftly diffuse to reach bacteria, forming micellar aggregates with bacterial membrane compositions. Static antimicrobial coatings, however, cannot freely diffuse, but rely on the movement of bacteria to form contact between their biocidal moieties and bacterial membranes.[10,24] Thus, difference in the relative performance of the GQ homologs in different settings may be magnified as the monomer units of the oligomeric surface coating have substantially fewer available conformations than the monomers in suspension to disrupt the target bacterial membrane.

The improved performance of GQ18 and GQ16 over MQA and MQB observed in the preceding tests may not be entirely

explainable with the suspension tests, as it is a highly idealized experimental setup. The lower micelle concentration of gemini surfactants is due to the second hydrophilic head that increases the available entropic states for water near the boundary. This may act as a key effect of the gemini element to reduce interfacial surface tension, enabling more uniform spread over the surface.

To test this hypothesis, droplets of GQ18, GQ16, and MQB were cast onto plane glass surface, and droplets of DW were applied on GQ18, GQ16, and MQB coated surfaces to measure contact angle formed between the surfaces and droplets. The lower DW droplet contact angles on the GQ coated surfaces compared to the conventional MQ coated ones suggest that GQs are more wettable. The higher surface energy of GQ solutions create is further correlated with their improved surface antimicrobial activities compared to MQ solutions.

It was hypothesized that covalent bonds between silanes to surfaces and other silanes and oligomerization all together increase coating durability by inhibiting water from solubilizing and removing the layer formed.[21,24,25] To test this, glass slides were coated with GQ18 and GQ16 and swabbed with a wet or dry cotton swab at a pressure of 8.8 kPa for 5 seconds for 10 times. After abrasion was applied, DW droplet contact angle was measured. These surfaces

were then inoculated with *S. aureus* and *E. coli* based on ISO 22196 to test for durability of antimicrobial efficacy after abrasion. Considering the hydrophilic nature of GQ solutions, it is suggestive that bonds between GQ compounds and surface complexes are more prone to removal by wet swabs. Although the increases in contact angles indicate the removal GQ compounds at some weakly bonded areas, no reduction in surface antimicrobial efficacies was observed.

Silanol containing monomers form durable coatings by initially forming hydrogen bonds with OH of the surface. Then, silanol monomers gradually undergo condensation and oligomerize with other silanol monomers and surface oxides, forming higher weight polysiloxanes and covalent surface linkages.[24,25] Thus, while GQ and MQ compounds are soluble in dilute aqueous solutions, it was hypothesized that aging the surface coating may allow substantially high degree of oligomerization and covalent surface bonds to form, hindering the ability of solvents to rinse away the coating.

To compare the durability and covalent nature of the GQ solutions with the conventional MQB, two sets of glass slides were prepared. The first group was coated with MQB, GQ18, and GQ16 and dried for 24 hours while the other group was first coated and then sequentially rinsed with MeOH and water. The FTIR spectra

were obtained by stretching vibration at 2850−2950 cm^{-1} which is diagnostic for the presence of organic compounds having sp^3 alkyl C−H bonds. Consistent with contact angle measurements and surface antimicrobial activity tests, FTIR spectra of GQ coated slides indicated a higher degree of deposition compared to the conventional MQ solution that showed no alkyl C−H.

Although the alkyl C−H stretches were reduced significantly on the surfaces treated with GQ18 and GQ16 after several rounds of rinsing, GQ surfaces still exhibited observable alkyl C−H stretches. This suggests highly adherent coatings formed by GQ molecules, despite they are soluble in both the MeOH and water. Silanol−containing monomers initially adhere to surfaces through Si−O−surface hydrogen bonding that gradually oligomerize, resulting in covalent surface linkages. The observed solvent and water−resistance may be due to this oligomerization that produces higher weight polysiloxanes and reduces oligomer solubility.

Residual antimicrobial efficacy of GQ solutions on three different types of non−porous surfaces after repeated contamination was examined. This was to simulate multiple contaminations which may occur in real−world scenario. The previous results had indicated gram−specific efficacy of GQ compounds, GQ18 against *S. aureus* and GQ16 against *E. coli*. The

results suggested that on all three types of non-porous surfaces, GQ coatings retained antimicrobial activity regardless of re-contamination.

Cell viability against GQ solutions was further examined with Mouse L929 fibroblasts to inspect cytotoxicity that may limit their use. At 0.1%, the concentration at which all tests were conducted, GQ16 showed 74.65956% cell viability, whereas MQB showed 21.71799% and GQ18 showing 27.52789%. The higher safety margin of GQ solutions over MQB and GQ16 over GQ18 may be due to their difference in hydrophobicity and hydrophilicity. As number of charges increases, alkyl chain lengths decrease, limiting diffusion into the cells.

5. Conclusion

In this study, a new class of surface modifying disinfectants "GQs" derived from gemini diquaternary organosilanes was developed and evaluated for antimicrobial and antiviral efficacy. Applied as dilute aqueous solutions, GQs showed efficacy against both gram-positive and negative bacteria on porous and non-porous surfaces, requiring only a short amount of time to be effective. GQ18 also showed efficacy against SARS-CoV-2. While conventional disinfectants and MQ solutions showed decreasing or no antimicrobial efficacy over time starting immediately after 5 minutes of application, GQs achieved almost 99.9999% reductions for 24 h and up to 15 days. Durability of GQ coatings is supported by results showing resistance to removal by solvents, with high residual antimicrobial efficacy after extended aging of up to 15 days, successive wet and dry abrasion, and repeated contamination. These results suggest GQs as promising agent to overcome the limitations of conventional disinfectants by providing immediate surface disinfection with long-lasting and durable antimicrobial efficacy. The findings from this study might benefit from further research on the possibility of GQ's application on the human body, for example, their effect in oral environment.

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containing quaternary ammonium salt groups. Biofouling 2008, 24 (3), 185-200.

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전염성 감염병의
접촉 전파 방지를 위한
제미니형 자가 표면 항균제 연구

서울대학교 치의학대학원 치의과학과
치과생체재료과학 전공

지도교수 안진수

김 민 주

연구목적: 본 연구에서는 다수의 소수성 꼬리와 2개 이상의 친수성 머리를 가진 새로운 제미니형 이-4차 암모늄 유기실란 항균제를 합성해 적용 가능 병원체의 범위가 넓고 발현이 빠르며 인체 유해성이 낮아 기존 항균제의 단점과 한계점을 극복한 항균제 연구를 실시했다.

연구대상 및 방법: 각각의 알킬기의 길이가 다른 세 종류의 제미니형 이-4차 암모늄 유기실란을 합성해 GQ18, GQ16, 그리고 GQ14라 명명했다. 대표적인 그람양성 세균인 *Staphylococcus aureus* (*S. aureus*)와 그람음성 세균인 *Escherichia coli* (*E. coli*) 등에 적용해 병원체에 대한 항균성을 평가했다. 항균제 현탁액에 병원체 직접 노출 후의 항균 효과, 비다공성 유리 표면과 다공성 네오프렌천에서의 항균 효과를 측정했다. 항균제 처리된 비다공성 표면에 물리적 마찰을 가한 후 증류수 방울이

이루는 표면각과 24시간 간격으로 병원체를 3번 연속 감염시킨 비다공성 표면에서의 항균제의 지속성을 측정했다. 또 항균제를 L929 섬유아세포에 적용해 세포독성 시험을 했다.

결과: 새로 합성한 액상 GQ18, GQ16, 그리고 GQ14 모두 그람음성 병원체를 직접 노출시켰을 때 15초 후 99.9999%의 항균성을 보였고 GQ18는 그람양성 병원체에도 비슷한 항균성을 보였다. 비다공성 표면에서 특히 GQ18은 *S. aureus*에 대해 15일 후에도 99.9999%의 항균성을, GQ16은 *E. coli*에 대해 15일 후에도 99.9999%의 항균성을 유지했다. 다공성 표면에서는 세 가지 항균제 모두 *S. aureus*에 대해 24시간 동안 99.9999%의 항균성을 보였고 *E. coli*에 대해서는 오직 GQ16만이 24시간 후에도 99.9999%동안 항균성을 보였다. 24시간 간격으로 3번 연속 병원체를 비다공성 표면에 적용한 후에도 GQ18은 S. aureus에, GQ16은 E. coli에 99.99~99.9999%의 항균성을 유지했다.

결론: 새로 합성한 제미니형 이-4차 암모늄 유기실란 항균제는 접촉 시 수 초 이내에 신속하게 항균성이 나타나 물리적 마찰에도 항균성을 오래 유지함을 알 수 있었다. 이 새로운 제미니형 이-4차 암모늄 유기실란 항균제는 병원체 접촉전파에 효과적인 세정제와 표면처리제로써의 활용과 개발 가능성을 보인다.

주요어: 접촉전파, 살균제, 세정제, 4차 암모늄, 제미니형 계면활성제, 항균성

학 번: 2018-34220