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Adhesion of *Aeromonas hydrophila* to Glass Surfaces Modified with Organosilanes

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Summary

The aim of this research is to study the adhesive properties of *Aeromonas hydrophila* to glass surfaces modified using four silanes with different reactive groups, namely (3-glycidoxypropyl)diethoxysilane, (3-N,N-dimethyl-3-N-n-hexadecylammoniopropyl)trimethoxysilane chloride, (3-N,N,N-triethanolammoniopropyl)trimethoxysilane chloride, and (3-N,N-dimethyl-3-N-n-octylammoniopropyl)trimethoxysilane chloride. The strain used in the study was *A. hydrophila* LOCK0968, isolated from the unchlorinated communal water distribution system in Poland. The effect of glass modification after chemical treatment was analyzed using surface tension measurement. The adhesive properties of the bacteria were studied in a water environment with a low concentration of organic compounds, using luminometric and microscopic methods. Additionally, the viability of the adherent bacterial cells was evaluated by counting the colony-forming units. The presence of active compounds in the culture medium after incubation with a modified carrier was verified using the Kirby-Bauer method. Half of the chemically modified glass surfaces exhibited better characteristics in comparison with native glass. Among the examined modifying agents, (3-N,N,N-triethanolammoniopropyl)trimethoxysilane chloride and (3-N,N-dimethyl-3-N-n-octylammoniopropyl)trimethoxysilane chloride showed the best antiadhesive and antibacterial properties. The most effective glass modification, with (3-N,N,N-triethanolammoniopropyl)trimethoxysilane chloride, was able to reduce the bacterial cell count by more than three orders of magnitude. The carriers had no significant effect on the viability of the free bacterial cells in the culture medium. Therefore, it can be said that the modified glass surface alone accounts for the antibacterial activity of the active organosilanes.

Key words: Aeromonas hydrophila, antiadhesion, glass, silanes

Introduction

Aeromonas hydrophila are facultatively anaerobic, oxidase-positive, Gram-negative, non-sporulating rods with rounded ends and with a length of 1–3.5 μm (*1*). Some species of *Aeromonas* genera are pathogenic to animals. Moreover, many strains are significant human pathogens and cause infections in various organs. The virulence factors appear in two forms: cell-associated structures and extracellular products. Among the cell-associated structures are pili, flagella, outer membrane proteins, lipopolysaccharides and capsules. Major extracellular products include cytotoxic, cytolytic, haemolytic, and enterotoxic proteins (*2*). Among aeromonads, *A. hydrophila* strains are clinically most significant. Recent studies have identified the presence of two Shiga toxin genes, *stx1* and *stx2*, in *Aeromonas* spp. that were isolated from clinical samples of patiensts with diarrhoea (*3*). According to the World Health Organisation's Guidelines for Drinking Water Quality, *A. hydrophila* is listed as a potential waterborne pathogen that causes diarrhoeal disease, wound infections, septicaemia, meningitis, oph-

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thalmitis, endocarditis, aspiration pneumonia and biliary tract infections (*4*).

Aeromonas spp. are found worldwide in different aquatic environments, including drinking water. The typical number of *Aeromonas* species in water distribution systems is between <1 and 10^3 CFU/mL. This number increases during summer periods and correlates with an increased number of diseases caused by aeromonads (*2,4–11*).

Growth rates of *Aeromonas* spp. cells are mostly affected by environmental conditions, such as nutrient availability, temperature and water activity. The growth rate is optimal at 30 °C, pH=7 and when water activity is 0.99 (*12*). Studies of low-nutrient waters showed that the bacteria can remain viable for extended periods of time. *Aeromonas* spp. can metabolize a wide variety of biopolymers, including proteins, carbohydrates and lipids. Only one microgram of amino acid and/or long chain fatty acid per litre can promote their growth in water distribution systems. The heterotrophic plate count (HPC) demonstrates that aeromonads can make up to 1 to 27 % of the total bacteria in the samples of drinking water (*13*).

Cell starvation in water environments is usually correlated with the adhesion of *Aeromonas* cells to different surfaces. *Aeromonas* spp. cells adhere to materials used in distribution systems such as stainless steel, copper, polybutylene and rubber. They are therefore able to colonise surfaces in water systems, forming biofilms (*2,4*). The formation of biofilms can be viewed as a survival mechanism and as a form of protection from toxic compounds, thermal stress and predators (*14,15*). Lack of nutrients induces physiological changes that reduce cell metabolism. Biofilm-growing bacterial cells live in unfavourable microenvironmental conditions and may undergo transformation into slow metabolic forms, including the viable but non-culturable (VBNC) state (*16*). Therefore, cell starvation may reduce the accuracy of classic culture methods used to detect bacteria.

Biofilms in water distribution systems may contain not only aeromonads, but also *Escherichia coli*, *Pseudomonas aeruginosa* or other pathogenic bacteria, and they play an important role in the transmission of various pathogens. In addition, microbial adhesion and biofilms in water systems contribute to the contamination of drinking water, reducing its quality and microbiological safety, and increasing the rate of corrosion in pipes. The reasons for using different antimicrobials include: (*i*) prevention of microbial contamination, deterioration, and preservation of process solutions and finished goods, (*ii*) reduction in the number of microorganisms that cause musty, mildew, and putrid odours, (*iii*) avoidance of aesthetic issues associated with mould and mildew stains, and (*iv*) reduced risk associated with medical conditions caused by microorganism transfer from medical devices (*14,17*).

The initial and key step leading to biofilm formation is bacterial adhesion to surfaces. This is a complex process, influenced by various physical and chemical properties of organisms, media and surfaces. Among these factors, only solid surface properties could be changed artificially in order to prevent biofouling in drinking water systems (*18*).

A significant number of studies have focused on surface modification. The universal antimicrobial surface should be environmentally neutral (without affecting the physical and chemical parameters of the environment), and not be used by microorganisms. Additionally, all microbial cells on this surface have to be destroyed completely to prevent the mutation of surviving cells. Traditional techniques involve the design of coatings with different biocidal agents, including antibiotics, quaternary ammonium salts, silver, *etc*. The antimicrobial treatment of surfaces is an interesting topic for the food and medical industry. Recently, different water-insoluble antibacterial materials with active groups immobilized on solid surfaces have been developed (*19–21*). Often such materials are siloxane-based polymers with biocidal groups bonded chemically to their chains. These can inhibit the growth of microorganisms without releasing toxic products of low molecular mass into the environment. When attached covalently to the surface of a variety of materials, they are able to kill bacteria through contact, and their antimicrobial activity is long-lasting and sustainable. Organofunctional silanes can be used for such surface modification. Silanes are silicon chemicals that possess a hydrolytically sensitive centre which reacts with inorganic substrates and forms stable covalent bonds. Organosilanes have a minimum of one carbon-silicon bond, *e.g.* Si-CH_3. This connection is very stable and nonpolar, and in the presence of an alkyl group it causes an increase in low surface energy and its hydrophobic qualities. Many combinations are possible, and may contain different types of reactive groups: methoxy, ethoxy or acetoxy, epoxy, amino, methacryloxy, chloro or sulphide (*22,23*). Silanization usually decreases the roughness of native surfaces, and this fact may be behind the increased anti-adhesive properties of modified surfaces (*24*).

Research into glass/water systems is important in terms of both the advancement of theoretical knowledge and practical applications. Although glass is widely believed to be an 'inert' material, glass surfaces are slightly acidic and highly adsorptive, due to the presence of silanol groups (SiOH) (*25*). These reactive groups interact *via* hydrogen bonding with other functional groups, allowing them to bond with the glass surface. These properties were used to impart new antibacterial properties to the native layers of the glass surface, in combination with different active organosilanes chosen for their excellent resistance to the combined effects of key environmental factors: water, heat and ultraviolet light (*22*). In this study, glass surfaces modified with different organosilanes are tested using *A. hydrophila* LOCK105 strain with strong adhesion qualities.

Materials and Methods

Bacterial strain and cultures

The study used *Aeromonas hydrophila* LOCK0968 isolated from the communal water distribution system in Poland (*11*). This strain was stored on antibiotic agar No. 1 (Merck, Darmstadt, Germany) slants at 4 °C under

standard laboratory conditions. For shake cultures, 50--fold diluted buffered tryptone water (Merck) was prepared with 200 mg/L of peptone. Then, 20 mL of buffered peptone water were poured into 25-mL Erlenmeyer flasks and covered with a cloth fixed with a rubber band. Sterile carriers were placed vertically in a liquid culture medium in such a way that half of the carrier was immersed in the medium, and the other part was above the liquid. The amount of inoculum was standardized to obtain a cell concentration in the culture medium equal to approx. 10–100 CFU/mL at the beginning of the experiment. The samples were incubated at 15 °C on a laboratory shaker (75 rpm) for 10 days.

Carriers

Star Frost (Knittel Glass, Bielefeld, Germany) 73×26 mm white glass slides were used as the reference material. The surface of the glass plates was modified with (3-glycidoxypropyl)diethoxysilane (G1), (3-N,N-dimethyl--3-N-n-hexadecylammoniopropyl)trimethoxysilane chloride (G2), (3-N,N,N-triethanolammoniopropyl)trimethoxysilane chloride (G3), and (3-N,N-dimethyl-3-N-n-octylammoniopropyl)trimethoxysilane chloride (G4) (Table 1).

Reagent grade chemicals N,N-dimethyl-N-hexadecylamine 95.0 %, N,N-dimethyl-N-octylamine 99.0 %, Tris(2-hydroxyethyl)amine 99.5 % (all from Sigma-Aldrich, St. Louis, MO, USA), (3-glycidoxypropyl)methyldiethoxysilane 97.0 % and (3-chloropropyl)trimethoxysilane 97.0 % (both from ACBR, Karlsruhe, Germany) were used without purification. Dimethylformamide (DMF), produced by POCH (Gliwice, Poland), was dried over $MgSO_4$ for 1 day and distilled under reduced pressure. Alkoxysilane with quaternary ammonium groups was prepared by reacting 3-chloropropylmethyldimethoxysilane with alkylamine in dry DMF at 45 °C for 48 h. The DMF and excess amine were removed by distillation using a high vacuum line. The yield from the substitution of chlorine reached 96–99 % as confirmed by proton nuclear magnetic resonance (^{1}H NMR) (*23*).

In the first step of this procedure the glass plates were immersed for 1 h in a solution of KOH/isopropanol (1:1) to remove any impurities. They were then rinsed with distilled water, immersed for 10 min in phosphoric acid solution (10 %), and rinsed again with distilled water. The chemically modified surfaces were prepared by the Center of Molecular and Macromolecular Studies of the Polish Academy of Sciences (CBMiM PAN, Lodz, Poland). The carriers were sterilized in 70 % ethanol for 24 h followed by a UV irradiation (λ=265 nm) for 1 h on each side.

Determination of contact angle and surface tension

In order to identify any changes that had occurred on the modified surfaces, contact angle measurements were taken. Determining the contact angle values for the two different solvents, dimethylformamide (DMF) and water, enabled the surface energy to be calculated. All measurements were performed using a ramé-hart NRL goniometer (ramé-hart instrument co., Succasunna, NJ, USA) equipped with a JVC KYF-70B camera (JVC, Yokohama, Japan). The dynamic contact angle was calculated using the DROPimage software (ramé-hart instrument co.), as the average of 15 measurements. The total surface tension was calculated from the values of the contact angles of the solvents of different polarity (Owens-Wendt method) (*23*).

Bacterial adhesion studies

The analysis of bacterial adhesion to the carriers was performed by luminometric measurement, the plate count method and microscopic observations. For the luminometric tests, the carrier plate was removed from the culture medium, rinsed with sterile distilled water and swabbed using sampling pens (Merck) free from adenosine triphosphate (ATP). The measurement was made in relative light units (RLU) using a HY-LiTE® 2 luminometer (Merck) (*11*).

For the determination of the colony-forming units (CFU), the carrier plate was removed from the culture medium, rinsed with sterile distilled water and swabbed using sterile swabs for surface testing. The bacterial suspensions were transferred onto a plate count agar (PCA; Merck), and after incubation at 25 °C for 48 h the colonies were counted. The number of colony-forming units per mL (of liquid media) or per cm^2 (of carriers) was calculated and presented as a logarithmic function.

In the microscopic studies, *A. hydrophila* cells were stained with basic fuchsin (0.5 %; Merck). Bacterial cells on the carrier were observed using the OLYMPUS BX41 light microscope with digital camera DP72 (Olympus, Center Valley, PA, USA). To evaluate the total cell adhesion area in the observation field, we used Image Tool v. 3.00 (UTHSCSA, San Antonio, TX, USA) (*26*).

Viability of bacterial cells in the culture medium and the assessment of surface chemical stability

To determine the number of free viable bacterial cells after 10 days of incubation on the tested surface in a culture medium, the CFU method and PCA medium (Merck) were used. Colonies of *A. hydrophila* were counted after 48 h of incubation at 25 °C and CFU/mL were presented as a logarithmic function.

Table 1. The chemical modifications of glass surface

Symbol	Active compound	Formula
G1	(3-glycidoxypropyl)diethoxysilane	$(CH_3CH_2O)_2CH_3Si(CH_2)_3OCH_2(CHCH_2O)$
G2	(3-N,N-dimethyl-3-N-n-hexadecylammoniopropyl)trimethoxysilane chloride	$[(CH_3O)_3Si(CH_2)_3(CH_3)_2N{+}C_{16}H_{33}]Cl^-$
G3	(3-N,N,N-triethanolammoniopropyl)trimethoxysilane chloride	$[(CH_3O)_3Si(CH_2)_3(CH_3)_2N{+}(CH_2CH_2OH)_3]Cl^-$
G4	(3-N,N-dimethyl-3-N-n-octylammoniopropyl)trimethoxysilane chloride	$[(CH_3O)_3Si(CH_2)_3(CH_3)_2N{+}C_8H_{17}]Cl^-$

The chemical stability of native and modified surfaces was determined after 10 days of incubating the carriers in a sterile culture medium. For this, the Kirby-Bauer test, also known as the disk diffusion method, was used. This method relies on the inhibition of bacterial growth measured under standard conditions. For this test, a culture medium, tryptic soy agar (TSA; Merck), was uniformly and aseptically inoculated with *A. hydrophila*. The filter paper discs were impregnated with the 10-day-old liquid medium and placed on TSA surface. When the active compound was present in the culture medium at a concentration at least equal to the minimal inhibitory concentration, a characteristic zone of inhibition was observed around the paper disc. These experiments were conducted in triplicate, and the standard deviation was calculated.

Results and Discussion

Surface tension

Surface tension is one of the most important physicochemical properties of any solid surface, and is correlated with resistance to biofilm formation. Fig. 1 presents the results of surface tension measurements on the tested surfaces, both native and after chemical modifications. The presence of active groups had an impact on the surface energy of the tested materials. The glass material modified with active organosilanes exhibited 50–67 % lower surface tension than the native glass surface. It was observed that the presence of organosilanes resulted in a significant decrease in the polar forces contributing to the surface energy of the materials. These facts are in a good agreement with the previous reports indicating that a hydrophobic film is formed on surfaces modified with silanes (*22*).

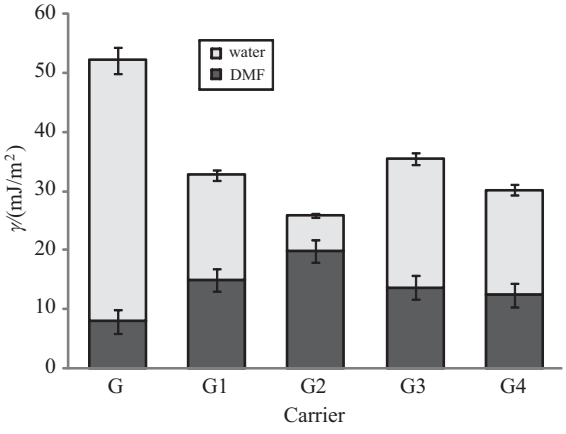

Fig. 1. Surface tension (γ) of glass (G) and its modifications (G1–G4). DMF=dimethylformamide

Bacterial adhesion

Since the number of *Aeromonas* cells in the water distribution systems that are poor in organic matter usually does not exceed 10^3 cells/mL, we used a minimal culture medium (containing 200 mg/L of peptone only), relatively long incubation time (10 days), and an initially very small cell concentration in the culture medium (10^1–10^2 CFU/mL). These conditions simulated the natural environment in which bacterial attachment and biofilm formation take place.

To determine the level of bacterial adhesion and viability of adhered cells, two main analytical methods, namely luminometry and plate count were used. Both methods require scraping the bacterial biofilm – a difficult procedure because biofilms are resilient, adherent and quite resistant to stripping by swabs. Additionally, swabbing may give misleading results because of the difficulties involved in obtaining a homogeneous cell suspension. The use of various procedures supporting 'disruption' (chemicals, ultrasound, laser shockwaves) is usually laborious and may be associated with a reduction in cell viability (*27–29*). It is worth noting that the plate count technique, in particular, requires that bacteria should remain culturable after the release process. Due to the difficulty of obtaining a homogeneous suspension after swabbing, the additional analytical method of microscopy was used.

Of course, there are different methods that can be used to test the antiadhesive properties of a surface, but there is no perfect test method (*30*). The rapid and simple luminometric method used in this study allowed the estimation of biological material on the test surfaces. This approach is based on bacterial ATP quantification and can be used not only to evaluate the total number of adhering cells, but also the cell viability of the bacteria that are unable to grow. This is worth noting, especially in the case of modified surfaces with antibacterial activity (*31–33*).

Fig. 2 presents the results of the luminescence test (in log RLU per cm^2) for all materials. The modified glass surfaces showed various levels of cell adhesion. For unmodified surfaces, the values were equal to 3.94 log RLU/cm^2. Strong antiadhesive properties were found in the case of surfaces G3 and G4. For these materials, the luminometric results of adhesion were 1.64 and 1.60 log RLU/cm^2, respectively.

The number of living bacterial cells on the native glass surfaces after 10 days of incubation was $6.1 \cdot 10^8$ CFU/cm^2 (Fig. 3). Half of the glass modifications con-

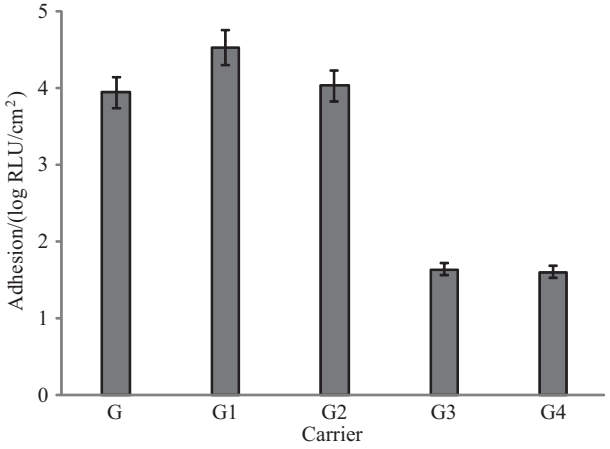

Fig. 2. Adhesion of *Aeromonas hydrophila* cells to glass carriers (G=unmodified and G1–G4=modified glass surfaces) measured by the luminescence method. RLU=relative light units

tributed to a decrease in the number of viable adherent *A. hydrophila*. The most effective antibacterial modification occurred in the cases of G3 and G4 modifications, which led to a significant reduction (three log units) in the number of adherent *A. hydrophila* cells.

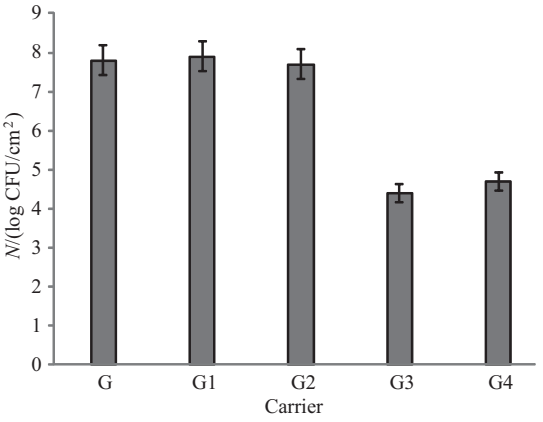

Fig. 3. Viability of adhered bacterial cells. G=unmodified and G1–G4=modified glass surfaces

In these experiments qualitative analysis of the adherent cells was used, based on light microscopy. A major problem in such evaluations of adhesion is the choice of stain for the cells. In the case of transparent glass surfaces, basic fuchsin can be used. Fig. 4 shows images of native and modified (with G3) glass surfaces. Irregular cell adhesion on the surface was detected on the native material, resulting in surface coverage ranging from approx. 30 to 50 % of the total area.

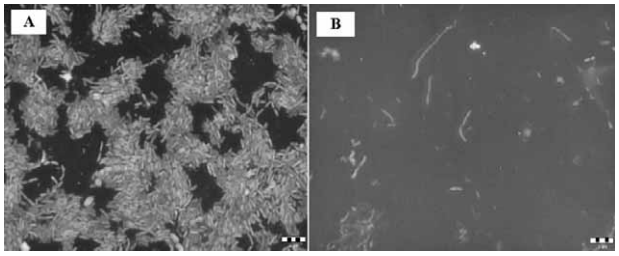

Fig. 4. Bacterial adhesion to (A) native and (B) glass surface modified with G3

The experiments revealed that glass surfaces modified with organosilanes G3 and G4 with active amino and methoxy groups can prevent the growth of *A. hydrophila.* These compounds contain two types of reactivity – methoxy group and amino group in the same molecule. Similar results were obtained with a modified surface coated with 3-(trimethoxysilyl)-propyldimethyloctadecylammonium chloride (*34*).

Viability of bacterial cells in culture medium and chemical stability of the carrier

The 10-day incubation of the bacterial suspension with the tested carriers was useful to verify not only the antimicrobial properties of the new materials, but also whether the modification was stable, *i.e.* if traces of substances inhibiting the growth of bacteria were present in the culture medium. The level of living bacterial cells in the culture medium after 10 days of incubation was 10^7–10^8 CFU/mL (Fig. 5). The carrier had no significant effect on the viability of free bacterial cells. Moreover, all of the modified active glass surfaces were found to be water stable. There were no active compounds in the medium at a concentration which inhibits the growth of *Aeromonas* spp. after 10 days of incubation (Fig. 6). These two facts mean that the antibacterial activity of the active compounds was associated only with the modified glass surface.

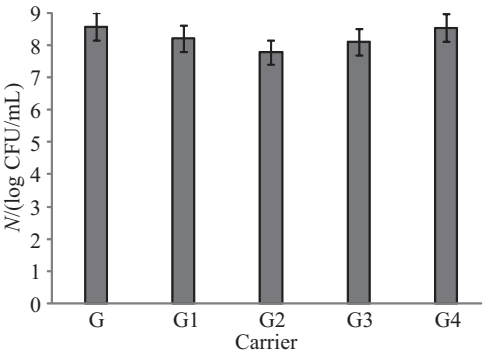

Fig. 5. Cell viability in the culture medium after a 10-day incubation with glass carrier. G=unmodified and G1–G4=modified glass surfaces

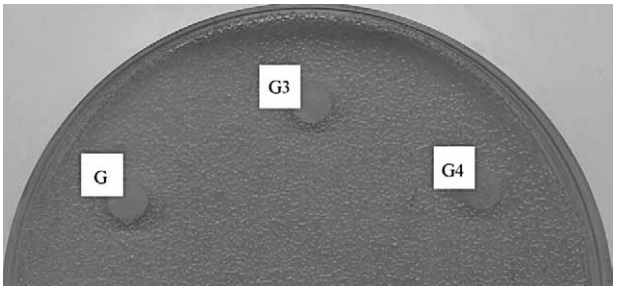

Fig. 6. Stability assessment of chemical modifications in the culture medium using Kirby-Bauer method. G=unmodified and G1 and G4=modified glass surfaces

Effect of material properties on bacterial adhesion

The general relationship between surface tension and the relative amount of adhesion was described as a 'Baier curve', and a zone of minimum adhesion was found in the region between 20 and 30 mJ/m^2 of the critical surface tension (*18*). Good bioadhesion occurs when this parameter exceeds 30 mJ/m^2. In this study, increasing the free energy on the material surface reduced the adhesion of *A. hydrophilia* significantly. This phenomenon is in accordance with the predictions of thermodynamics. In a study by Katsikogianni and Missirlis (*35*), the adhesion of bacterial strains was found to be dependent on the terminal functionality of the monolayer. For example, it was the highest on CH_3, followed by the positively charged NH_2, the non-charged NH_2 groups, the COOH and finally the lowest on the OH-terminated glass.

In this study, the results do not seem to be explained by the surface energy of the various substrates, as materials that present similar surface energy values had different antimicrobial performances (G1 and G4). Bull *et al*. (*36*) reported that methoxy, amino and chloro groups present high cytotoxicity and are often used in disinfection; therefore it seems that they play a crucial role in the inactivation of microorganisms. Although the molecular structures of G2 and G3 were very similar, the antibacterial performance of G3 was much better than that of G2. It may only be assumed that the antibacterial activity of a compound is also determined by the spatial orientation of its molecule. It has been confirmed that molecules that are not identical to a comparator – which have structural differences, spatial orientation, and/or impurities – can produce different antibacterial effects (*37*).

Conclusions

It is clear from a number of studies that *Aeromonas* sp. is an emerging player causing infectious disease. Biofilm formation by species of *Aeromonas* in water distribution systems can play a key role in the contamination of drinking water and transmission of different pathogens. Therefore, a significant problem is how to prevent the formation of biofilms. The best strategy for the food and water industries is to reduce microbial adhesion at the outset. The candidate materials for use in water distribution systems should detach and eliminate the initially adhered bacteria. Materials with (3-N,N,N--triethanolammoniopropyl)trimethoxysilane chloride, and (3-N,N-dimethyl-3-N-n-octylammoniopropyl)trimethoxy silane chloride (G3 and G4) seem promising as suitable coatings for materials that come into contact with drinking water, due to their significant antibacterial properties and their ability to repel pathogens implicated in the contamination of water.

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