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Full length article

Comparison of methods to evaluate bacterial contact-killing materials

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ABSTRACT

Cationic surfaces with alkylated quaternary-ammonium groups kill adhering bacteria upon contact by membrane disruption and are considered increasingly promising as a non-antibiotic based way to eradicate bacteria adhering to surfaces. However, reliable *in vitro* evaluation methods for bacterial contact-killing surfaces do not yet exist. More importantly, results of different evaluation methods are often conflicting. Therefore, we compared five methods to evaluate contact-killing surfaces. To this end, we have copolymerized quaternary-ammonium groups into diurethane dimethacrylate/glycerol dimethacrylate (UDMA/GDMA) and determined contact-killing efficacies against five different Gram-positive and Gram-negative strains. Spray-coating bacteria from an aerosol onto contact-killing surfaces followed by air-drying as well as ASTM E2149-13a (American Society for Testing and Materials) were found unsuitable, while the Petrifilm[®] system and JIS Z 2801 (Japanese Industrial Standards) were found to be excellent methods to evaluate bacterial contact-killing surfaces. It is recommended however, that these methods be used in combination with a zone of inhibition on agar assay to exclude that leakage of antimicrobials from the material interferes with the contact-killing ability of the surface.

Statement of Significance

Bacterial adhesion to surfaces of biomaterials implants can be life-threatening. Antimicrobials to treat biomaterial-associated infections often fail due to the bacterial biofilm-mode-of-growth or are ineffective due to antibiotic-resistance of causative organisms. Positively-charged, quaternized surfaces can kill bacteria upon contact and are promising as a non-antibiotic-based treatment of biomaterial-associated infections. Reliable methods to determine efficacies of contact-killing surfaces are lacking, however. Here, we show that three out of five methods compared, including an established ASTM, are unsuitable. Methods found suitable should be used in combination with a zone-of-inhibition-assay to establish absence of antimicrobial leaching, potentially interfering with contact-killing. Identification of suitable assays for evaluating bacterial contact-killing will greatly assist this emerging field as an alternative for antibiotic-based treatment of biomaterial-associated-infections.

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1. Introduction

Bacterial adhesion and subsequent biofilm formation can be a costly problem in many fields. Examples can be found in e.g. food processing and packaging industry, drinking water systems, in the marine environment, on surfaces exposed to a hospital environment, including dental restorative materials and the surfaces of

biomaterials implants and devices. Especially in the biomedical arena, bacterial adhesion can yield life-threatening diseases [1,2].

Different types of coatings are being considered as antibacterial or infection-resistant that are either non-adhesive to bacteria such as hydrophobic coatings [3,4], polyethylene glycol (PEG) brush coatings [5,6], hydrogel coatings [7], coatings with nanoparticles [8] or antibiotic releasing coatings [9], which are aimed to yield high particle or antibiotic concentrations around a biomaterials implant or device in order to kill the bacteria present [10]. A drawback of these 'release-killing' materials is, that they all show a high-burst release upon insertion in the human body, followed by a low-level tail-release that can extend to several years. Since

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the low-level tail-release often yields concentrations insufficient for killing but also far below the minimal inhibitory concentration for growth, tail-release has been associated with the development of antibiotic-resistant strains [11,12]. Polymers containing covalently bonded antimicrobial moieties, such as immobilized quaternary ammonium compounds, possess the unique feature of bacterial ‘contact-killing’ [13]. Provided the cationic charge density [14,15] on the surface is above 10^{14} positive charges per cm^2 and created through alkylated ammonium groups with appropriate alkyl chain lengths [16], adhering bacteria will be killed upon contact by severe membrane disruption through extremely strong electrostatic attraction [17]. Bacterial killing upon adhesion to cationic quaternary ammonium coated surfaces has been shown in many *in vitro* studies [13,14,17–25] while *in vivo* efficacy of cationic coatings has been demonstrated in rats [26] and sheep [27]. Bacterial contact-killing materials and coatings are increasingly promising as a non-antibiotic based way to eradicate bacteria adhering to surfaces, but largely confine themselves to coatings comprised of quaternized ammonium compounds with a suitable hydrocarbon tail length. Moreover, cell wall damage may often be so severe leaving little possibilities for adhering bacteria to stay alive in a growth inhibited state, whatever alive may mean for a bacterium [28].

Despite their promise, no ubiquitously accepted method to evaluate the efficacy of bacterial contact-killing of cationic surfaces exists. Often applied methods (see Table 1 for a description of the essential features of these methods) include the ASTM E2149-13a (American Society for Testing and Materials) [29], the JIS Z 2801 (Japanese Industrial Standards) [30] and the modified JIS method [31], spray-coating of bacteria on a surface from an aerosol [32] and the Petrifilm® assay [33]. A comparison of methods to establish bacterial contact-killing on cationic surfaces has never been made however, but is direly needed considering the interest in the topic, that is stimulated by the increasing lack of effective antimicrobials worldwide [34]. Therefore the aim of this study was to evaluate and compare five methods frequently used in the current literature with respect to their efficacy to evaluate bacterial contact-killing using different Gram-positive and Gram-negative bacterial strains. As an easy to prepare contact-killing material, quaternary ammonium groups were directly copolymerized into conventional diurethane dimethacrylate/glycerol dimethacrylate (UDMA/GDMA), yielding a fully crosslinked material with demonstrated ability to facilitate contact-killing of a variety of different bacterial strains in absence of leaching antibacterial compounds [18]. Bacterial strains used were *Escherichia coli*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Streptococcus mutans*, that all occur in a wide range of applications where bacterial adhesion to surfaces can be troublesome. Criteria to demonstrate bacterial death are not trivial unfortunately. Many living bacteria can be unculturable, while sometimes bacteria indicated to be dead by LIVE/DEAD staining appeared culturable [28]. Hence we used the criteria for cell death as given in the protocols of respective methods evaluated, with taking the ratio of the log reduction in viable organisms observed over the maximal log reduction that could be achieved considering the bacterial challenge applied in a certain method as the final criterion for comparison.

2. Materials and methods

2.1. Preparation of the positively charged, quaternary ammonium containing polymer samples

The preparation of positively charged quaternary ammonium polymer samples was described before in detail [18]. Briefly, UDMA (52 wt%), GDMA (35 wt%) and quaternary ammonium methacrylate with an alkyl chain length of C_{12} (QA- C_{12}) (13 wt%) were mixed and

sonicated at room temperature for 120 min to create a homogeneous solution. Subsequently, after complete dissolving, the photo-initiators camphorquinone (CQ) (0.5 wt% solution) and ethyl-4-dimethylaminobenzoate (EDMAB) (0.5 wt% solution) were added and sonication was performed for another 30 min to dissolve the photo-initiators in the mixture. As a control polymer, the mixture was also prepared without QA- C_{12} . Samples with a diameter of 15 mm and 0.5 mm thick were prepared using a polydimethylsiloxane mold. The mold was filled with the polymer, air bubbles were removed and a glass slide was placed on top of the mold in order to create a smooth surface. Light-curing (Optilux 501, Kerr Dental, Middleton, WI, USA) with an irradiance of around 1000 mW/cm^2 was performed on both sides for 90 s. After light-curing, samples were washed with isopropanol to remove unreacted monomers. All samples were sterilised by immersion in 70% ethanol followed by air drying. Prior to sterilization, UDMA/GDMA/QA- C_{12} samples were first kept for three days in 200 mL demineralized water per sample at 37°C , while refreshing the water every 24 h, in order to remove possible antibacterial leachables.

2.2. Characterization of the quaternary ammonium polymer samples

2.2.1. X-ray photoelectron spectroscopy (XPS)

Quaternized nitrogen on the sample surface was determined by XPS, as described before [35]. Briefly, an XPS (S-probe; Surface Science Instruments, Mountain View, CA), equipped with a monochromatic X-ray source (Al $K\alpha$ anode yielding 1486.8 eV X-rays), was operated at 10 kV accelerating voltage and 22 mA filament current. The direction of the photoelectron collection angle was set to 35° with respect to the sample surface, and the electron flood gun was set at 10 eV. A survey scan was made with a $1000 \times 250 \mu\text{m}^2$ spot and a pass energy of 150 eV. Binding energies were determined by setting the binding energy of the C_{1s} binding energy peak (carbon bound to carbon) at 284.8 eV . Detailed scans of the N_{1s} binding energy peaks over a binding energy range of 20 eV were made using a pass energy of 50 eV. The N_{1s} peak was subsequently decomposed in two fractions at 399.3 and 402.4 eV . The occurrence of a peak at 402.4 eV is indicative for the presence of quaternized nitrogen species [2] and was expressed in atom percentage (at.%) charged nitrogen species by multiplying the peak fraction at 402.4 eV with the total at.% nitrogen.

2.2.2. Cationic charge density using fluorescein staining

The cationic charge density of the sample surfaces was determined using fluorescein staining. To this end, UDMA/GDMA/QA- C_{12} and UDMA/GDMA control samples were immersed in 2 mL 1 wt% fluorescein (disodium salt) solution in demineralized water and shaken at 60 rpm for 10 min. The samples were washed three times with 2 mL demineralized water to remove any dye not complexed with cationic charges. Next, the samples were placed in 2 mL of a 0.1 wt% cetyltrimethylammonium chloride solution in demineralized water and sonicated for 5 min and shaken at 60 rpm for 5 min to desorb complexed fluorescein dye. Subsequently, 200 μL of 100 mM phosphate buffer, pH 8, was added. UV/VIS measurements (Spectra max M2 UV/VIS spectrophotometer) were carried out at 501 nm to yield the concentration of fluorescein dye in the extraction solution [Dye] in M according to

$$[\text{Dye}] = (\text{Abs}_{501}) / (\epsilon_{501} \times L) \quad (1)$$

in which Abs_{501} is the UV absorption at 501 nm, ϵ_{501} is the extinction coefficient ($77 \text{ mM}^{-1} \text{ cm}^{-1}$ for fluorescein) and L is the length of a polystyrene cuvette (1 cm) traversed by the UV-light beam. The cationic charge density per cm^2 sample surface area was subsequently calculated using:

$$\text{Charge density} = [\text{Dye}] \times V \times N/A \quad (2)$$

Table 1

Summary of the methods compared in this paper for the evaluation of the efficacy of contact-killing materials containing covalently bonded antimicrobial moieties, together with their advantages and disadvantages as perceived, based on the comparisons made in the current paper.

Method	Description	Advantages	Disadvantages	References
Petrifilm®	Bacteria on a contact-killing surface are confined between a transparent film containing nutrients and a staining agent and allowed to grow for a defined time period	Small fluid volume ensuring contact between bacteria and sample surface Nutrient availability during the experiment No additional steps for <i>in situ</i> enumeration	High numbers of bacteria cannot be counted Antibacterial leachables cannot be excluded	[17,18]
JIS Z 2801	A droplet of a bacterial suspension in buffer is placed on a contact-killing surface and left to incubate for a defined time period	Small fluid volume ensuring contact	No nutrient availability during the experiment Antibacterial leachables cannot be excluded Bacteria have to be dislodged for enumeration	[19]
Modified JIS	A bacterially inoculated filter is placed on a contact-killing surface, and placed with the filter side on a nutrient agar	Small fluid volume ensuring contact. Nutrient availability during the experiment	Bacteria may reside deep in the filter and not come in contact with the sample Antibacterial leachables cannot be excluded Bacteria have to be dislodged for enumeration High nutrient availability stimulates bacterial growth also on contact-killing surfaces	[20]
Bacterial spray	Bacteria are sprayed from an aerosol to a contact-killing surface	Mimics bacterial contamination of surfaces through transport in air	Challenge number difficult to determine Dehydration will contribute to cell death	[13,21,32]
ASTM E2149	Incubation of a surface in a bacterial suspension while shaking	Flexible with regards to shape and size of substratum	No nutrient availability during the experiment Antibacterial leachables cannot be excluded Challenge number unknown and dependent on shaking, i.e. number of collisions between bacteria and sample surface Enumeration of dead bacteria indirect	[14,22–24]

in which V is the volume of the extraction solution (1 mL), N is Avogadro's number (6.023×10^{23}) and A is the surface area of the UDMA/GDMA/QA-C₁₂ polymer sample (3.77 cm²).

2.2.3. Assessing leaching of antibacterial components

In order to determine whether after three days immersion of samples in water, antibacterial components leached out in antibacterially active amounts, Brain Heart Infusion (OXOID, Basingstoke, UK) agar plates were inoculated with a suspension of 1×10^8 bacteria per mL of *Enterococcus faecalis* OG1RF with a cotton swab and air dried for 2 min. The washed samples were placed in the middle of the agar plates and incubated for 24 h at 37 °C. The plates were assessed for the development of an inhibition zone surrounding the UDMA/GDMA/QA-C₁₂.

2.3. Bacterial strains, grow conditions and harvesting

The bacterial strains used in this study were Gram-negative *E. coli* ATCC25922 and Gram-positive *S. epidermidis* ATCC12228,

E. faecalis OG1RF, *S. aureus* ATCC12600 and *S. mutans* NS. The bacterial strain was first streaked on a blood agar plate from a frozen stock solution (7 v/v% DMSO) and grown overnight at 37 °C. One colony was inoculated in 10 mL of the appropriate growth medium and incubated at 37 °C for 24 h. The growth media used were: Brain Heart Infusion for *E. coli* and *E. faecalis*, Tryptone Soya Broth (OXOID) for *S. epidermidis* and *S. aureus*, and Todd Hewitt Broth (OXOID) for *S. mutans*. 10 mL of these pre-cultures was used to inoculate a main culture of 200 mL growth medium, which was incubated for 18 h at 37 °C. Bacteria were harvested by centrifugation for 5 min at 6500g and 10 °C three times and subsequently washed with phosphate-buffered saline (PBS; 10 mM potassium phosphate and 150 mM NaCl, pH 7.0). The number of bacteria in suspension were enumerated using a Bürker-Türk counting chamber. Concentrations were adjusted by dilution with PBS to the requirements of the specific methods. The percentage live bacteria in a suspension was determined by colony forming unit (CFU) counting.

2.4. Methods for evaluating bacterial killing upon contact with the surface

All evaluation methods were applied in triplicate with separately grown bacterial cultures. In order to provide for a comparable scale valid for all five methods included in this study, we have determined the maximum log-reduction in the number of live or viable bacteria that could be achieved in each method. Next, the actual log-reduction achieved in each method was determined according to

$$\text{Log reduction} = \log_{10}(\text{challenge number per cm}^2) - \log_{10}(\text{viable number per cm}^2 \text{ on contact killing surface}) \quad (3)$$

and expressed as a 'percentage contact-killing efficacy' according to

$$\text{Efficacy} = \frac{\text{Log reduction}}{\text{Max log reduction}} 100\% \quad (4)$$

Adsorbed protein film. A macromolecular conditioning film was applied on the samples in order to perform the evaluations of contact-killing efficacies under more practically realistic conditions.

For possible oral applications, this involved adsorption of a salivary film from lyophilized human whole saliva [36], reconstituted in 1.5 mg/mL buffer (2 mM potassium phosphate, 1 mM CaCl₂, 50 mM KCl, pH 6.8). Each sample was immersed overnight in 2.5 mL of thus prepared saliva, taken out with a pair of sterile tweezers, and the excess saliva was dripped off before conducting the evaluation of their contact-killing efficacy against the *S. mutans* strain employed, an oral pathogen. Similarly, samples were immersed in 10% fetal calf serum and evaluations performed against an *S. epidermidis* strain, often found on biomaterial implants in the human body.

2.4.1. Petrifilm® method

The Petrifilm® Aerobic Count plate system (3 M Microbiology, St. Paul, MN, USA) [33] consists of two films: a bottom film containing standard nutrients, a cold-water gelling agent and an indicator dye that facilitates colony counting and a top film enclosing the sample within the system. The bottom film containing the gelling-agent was first swelled with 1 mL sterile demineralized water for 60 min and transferred to the transparent top film before use. Next, a sterile UDMA/GDMA/QA-C₁₂ and UDMA/GDMA sample were placed on the bottom film. 10 µL of different dilutions of bacterial suspensions (10⁴, 10⁶ and 10⁸ bacteria/mL) was pipetted on top of each samples and the top film was closed. Closure of the Petrifilm® system ensured spreading of the bacterial suspension over the surface area of the samples. The closed Petrifilm® system was left to incubate at 37 °C for 48 h after which the numbers of CFUs were counted and used to calculate the percentage contact-killing efficacy.

2.4.2. Japanese Industrial standard method (JIS)

In the JIS Z 2801:2010 [30], samples were placed in a sterile 6-wells plate. 10 µL of a bacterial suspensions in PBS (10⁸ bacteria/mL) was pipetted on top of a sterile UDMA/GDMA/QA-C₁₂ and UDMA/GDMA sample. Next, the well plate was covered with sterilized Parafilm® (diameter 15 mm) and left to incubate at 37 °C for 24 h under humidified atmosphere. After incubation, 5 mL 0.1% (v/v) Tween80 in PBS was added to each well, followed by sonication for 30 s and gentle shaking for 2 min in order to dislodge adhering bacteria. The resulting suspension was serially diluted and the numbers of CFUs were determined by plate counting on agar after 24 h incubation at 37 °C, from which the percentage contact-killing efficacy was calculated.

2.4.3. Modified JIS method

In the modified JIS method [31], 10 µL of a bacterial suspension in PBS (10⁸ bacteria/mL) was pipetted on sterilized nitrocellulose filters (pore size 0.45 µm and diameter 15 mm) placed on a agar plate. The liquid was absorbed by the agar while the bacteria retained on the filter. A sterile UDMA/GDMA/QA-C₁₂ and UDMA/GDMA sample were placed in a 6-wells plate. Next, 20 µL of the appropriate growth medium in 10 mM potassium phosphate buffer (1% v/v), with 50% fetal calf serum added, was pipetted centrally on the surface of a sample after which an inoculated filter was carefully placed on the sample, with filter-side on which the bacteria were retained contacting the sample. The system was left to incubate at 37 °C for 24 h in humidified atmosphere. After incubation, 5 mL growth medium was added to each sample and corresponding filter, sonicated for 30 s and vortexed for 1 min to dislodge adhering bacteria. Finally, the resulting suspensions were serially diluted and the numbers of CFUs were determined by plate counting on agar after 24 h incubation at 37 °C and used to calculate the percentage contact-killing efficacy.

2.4.4. Bacterial spray method

A bacterial suspension with a concentration of 1 × 10⁸ bacteria per mL was sprayed for 2 s onto a sterile UDMA/GDMA/QA-C₁₂, UDMA/GDMA sample and control glass slide from a distance of approximately 15 cm, placed under an angle of 45° [32]. After spraying, surfaces with adhering bacteria were air dried for 2 min and stained for 15 min in the dark with 15 µL live-dead BacLight (BacLight™, Molecular probes, Leiden, The Netherlands) viability stain containing SYTO 9 dye (yielding green fluorescence for live bacteria) and propidium iodide (yielding red fluorescence in cell membrane-damaged bacteria, generally considered to be "dead" bacteria). In order to ensure proper indication of dead organisms, we have used the SYTO 9/propidium iodide stain in a 1:1 concentration, as for some bacterial strains species higher amounts of propidium iodide are required, possibly with longer incubation times, as also described in the manufacturers manual. A fluorescence microscope (Leica DM4000B; Leica Microsystems GmbH Heidelberg, Germany) was used to visualize live and dead adhering bacteria. After enumeration of the number of viable and dead bacteria, the percentage of viable bacteria was determined and expressed as percentage contact-killing efficacy, as defined above.

2.4.5. ASTM method

In the ASTM E2149-13a [28] protocol, 10 mL of a bacterial suspension (3 × 10⁵ bacteria/mL) was added into test tubes together with or without sterile UDMA/GDMA/QA-C₁₂ and UDMA/GDMA samples and agitated in an orbital mixer at 200 rpm for 15 min at room temperature (21 °C). After 5, 10 and 15 min 100 µL aliquots were taken of the suspension, serially diluted and the numbers of CFUs were determined by plate counting on agar and used to calculate the percentage contact-killing efficacy. In one experiment, aliquots were taken up to 120 min in order to check whether contact-killing continued after 15 min.

2.4.6. Statistical analysis

Data were analyzed with the Statistical Package for Social Science (Version 16.0, SPSS Inc., Chicago, IL, USA). A Student *t*-test was used to compare the number of bacteria in log reduction between UDMA/GDMA (control) and UDMA/GDMA/QA-C₁₂ (contact killing material) of each bacterial strain and the mean of all five bacterial strains for each method evaluated. A one-way analysis of variance (ANOVA) was used to compare the killing efficiency on all five bacterial strains between different methods on UDMA/GDMA/QA-C₁₂. A Bonferroni test was used for post-hoc multiple comparisons. Statistical significance was set at *p* < 0.01.

Fig. 1. Characterization of cationically charged, UDMA/GDMA/QA_C12 samples. (a) UDMA/GDMA and UDMA/GDMA/QA_C12 sample. (b) Quantitative evaluation (averages with standard deviations over 5 samples) of N_{1s} photoelectron binding energy peaks of UDMA/GDMA and UDMA/GDMA/QA_C12. (c) N_{1s} photoelectron binding energy peak for UDMA/GDMA. (d) N_{1s} photoelectron binding energy peak for UDMA/GDMA/QA_C12, with a clear peak component at 402.4 eV, indicative of the incorporation of quaternary ammonium groups.

3. Results

3.1. Characterization of cationically charged, UDMA/GDMA/QA_C12 samples

The cationic charge density of the UDMA/GDMA/QA_C12 samples was quantified by fluorescein staining (Fig. 1a) and amounted $2.5 \times 10^{16} \text{ N}^+/\text{cm}^2$ well above 10^{14} positive charges per cm^2 required to kill bacteria upon contact [14,37]. XPS yielded a slight increase in the at.% N upon copolymerization with alkylated ammonium into UDMA/GDMA (Fig. 1b), but after copolymerization the N_{1s} photoelectron binding energy peak showed a clear component at 402.4 eV indicative of quaternized nitrogen (compare Fig. 1c and d) and confirming successful incorporation of positively charged quaternized nitrogen in the polymer matrix [35].

In order to exclude that leaching of antibacterial components out of the material prepared would interfere with their contact-killing, all samples were immersed for three days at 37 °C in an excess of demineralized water (200 mL per sample, refreshing the volume every 24 h). No antibacterial efficacy was observed by the lack of an inhibition zone around a disc shaped sample placed on a bacterially inoculated agar plate (Fig. 2). Moreover, only bacteria underneath the sample were killed, inferring contact-killing.

3.2. Methods for evaluating bacterial killing upon contact with the surface

All methods compared (Table 1) were applied as closely as possible to the instructions given in the authentic literature references or instruction guides. Minor modifications were made to keep the bacterial challenge numbers per cm^2 in each method approximately equal for better comparison. However, minor variations in

Fig. 2. Absence of an inhibition zone around an UDMA/GDMA/QA_C12 sample (diameter 15 mm) on an agar plate inoculated with *E. faecalis* OG1RF indicated absence of leaching of antibacterial components from the samples that might interfere with the evaluation of contact-killing. Note the absence of bacteria underneath the sample, inferring bacterial contact-killing.

bacterial challenge numbers could not be avoided and challenges ranged from $7.5 \times 10^4 \text{ CFU cm}^{-2}$ for ASTM E2149-13a to $5.8 \times 10^5 \text{ CFU cm}^{-2}$ for the bacterial spray assay (Table 2). The logarithms of these challenge numbers represent the maximal log

Table 2
Log reductions actually achieved in the various methods for five bacterial strains.^a For each method, the bacterial challenge applied is given per cm², from which the maximum log reduction that can possibly be achieved is calculated. $\pm$ Signs indicate the SD over triplicate experiments with separately cultured bacteria.

Method	Petrfilm®	JIS Z 2801	Modified JIS	Bacterial spray	ASTM E2149
Challenge (CFU cm ⁻²)	5×10^5	5×10^5	5×10^5	5.8×10^5	7.5×10^4
Max log reduction possible	5.7	5.7	5.7	5.8	4.9
UDMA/GDMA: log reduction achieved					
<i>E. coli</i> ATCC25922	0.1 $\pm$ 0.2	0.4 $\pm$ 0.3	-1.5 $\pm$ 0.5	0.0 $\pm$ 0.0	0.3 $\pm$ 0.2
<i>S. epidermidis</i> ATCC12228	-0.1 $\pm$ 0.2	0.5 $\pm$ 0.2	-1.9 $\pm$ 0.5	0.0 $\pm$ 0.0	0.4 $\pm$ 0.1
<i>E. faecalis</i> OG1RF	0.0 $\pm$ 0.0	0.1 $\pm$ 0.2	-0.6 $\pm$ 0.1	0.0 $\pm$ 0.0	0.6 $\pm$ 0.4
<i>S. aureus</i> ATCC12600	0.0 $\pm$ 0.0	-0.1 $\pm$ 0.1	-0.1 $\pm$ 0.1	0.1 $\pm$ 0.0	0.1 $\pm$ 0.3
<i>S. mutans</i> NS	0.0 $\pm$ 0.0	-0.1 $\pm$ 0.1	0.3 $\pm$ 0.4	0.1 $\pm$ 0.0	0.1 $\pm$ 0.3
UDMA/GDMA/QAC ₁₂ : log reduction achieved					
<i>E. coli</i> ATCC25922	1.0 $\pm$ 0.7	5.1 $\pm$ 1.1 ^a	-1.2 $\pm$ 1.1	0.0 $\pm$ 0.0	0.1 $\pm$ 0.2
<i>S. epidermidis</i> ATCC12228	5.7 $\pm$ 0.0 ^a	5.7 $\pm$ 0.0 ^a	5.7 $\pm$ 0.0 ^a	0.2 $\pm$ 0.2	0.2 $\pm$ 0.1
<i>E. faecalis</i> OG1RF	5.7 $\pm$ 0.0 ^a	5.7 $\pm$ 0.0 ^a	4.2 $\pm$ 1.4	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1
<i>S. aureus</i> ATCC12600	5.7 $\pm$ 0.0 ^a	5.7 $\pm$ 0.0 ^a	5.7 $\pm$ 0.0 ^a	0.1 $\pm$ 0.0	0.1 $\pm$ 0.3
<i>S. mutans</i> NS	5.7 $\pm$ 0.0 ^{a,b}	5.7 $\pm$ 0.0 ^{a,b}	3.7 $\pm$ 1.9 ^{b,c}	0.1 $\pm$ 0.0 ^d	0.1 $\pm$ 0.3 ^d

^a Negative log reductions indicate bacterial growth.

^a Significantly different from UDMA/GDMA tested on the same bacterial strain using the same method.

^b Significantly different from UDMA/GDMA tested on all five bacterial strains using the same method.

^c Significantly different between JIS Z 2801 and Modified JIS.

^d Significantly different from Petrfilm®, JIS Z 2801 and Modified JIS.

reduction that can be achieved in a particular method (see also Table 2).

In Table 2 it can be seen that none of the methods demonstrated contact-killing on control samples of UDMA/GDMA, and maximal log reductions of 0.6 CFU cm⁻² were observed for *E. faecalis* OG1RF on UDMA/GDMA in ASTM E2149-13a. Whereas in some methods, minor growth (negative log reductions) in the order of -0.1 CFU cm⁻² was observed on UDMA/GDMA samples, major growth was found in the modified JIS method, with abundant nutrient availability, for four out of the five strains used, even up to -1.9 CFU cm⁻² for *S. epidermidis* ATCC12228. Accordingly, the modified JIS method was the only method that exhibited growth for *E. coli* ATCC25922 on contact-killing UDMA/GDMA/QA_{C12} samples. The bacterial spray method and ASTM E2149-13a showed far lower log reductions than did the Petrfilm® and JIS Z 2801 methods ($p < 0.01$).

Fig. 3a compares the percentage contact-killing efficacy in the different methods, made directly comparable by defining the contact-killing efficacy in a method as the ratio over the log reduction observed over the maximal log reduction that could be achieved considering the bacterial challenge applied. If the entire bacterial challenge applied was killed, the efficacy of the method was denoted as 100%. Generally, the bacterial spray method and ASTM E2149-13a indicated absence of contact-killing, while the JIS Z 2801 and the Petrfilm® method indicated 100% contact-killing efficacy for four out of the five strains, with the exception of Gram-negative *E. coli* showing less contact-killing. The modified JIS method demonstrated near 100% contact-killing efficacy for *S. epidermidis* and *S. aureus*, but not for *E. faecalis* and *S. mutans*, while showing growth for the *E. coli* strain.

In the ASTM E2149-13a method, samples are agitated in 10 mL bacterial suspension during 15 min, after which the killing efficacy is determined. Considering the lack of contact-killing observed using ASTM E2149-13a, the method was also carried out with longer agitation times up to 60 min and 120 min, but this did not make any difference with respect to the ability of the method to demonstrate bacterial contact-killing (see Supplementary Fig. 1).

In many potential applications of contact-killing coatings, the coatings attract a film of adsorbed macromolecular components before bacteria adhere. Under clinical conditions in the human body for instance, biomaterial surfaces often become coated with a layer of proteins adsorbed from body fluids before the first bacteria adhere. Hence, to enlarge the practical significance of our

comparison, UDMA/GDMA/QA_{C12} samples were first provided with a film of adsorbed salivary proteins and subsequently evaluated against the oral bacterium *S. mutans* NS or a film of proteins adsorbed from 10% fetal calf serum and evaluated against *S. epidermidis* ATCC12228. The killing efficacy of UDMA/GDMA/QA_{C12} was not affected by the presence of an adsorbed macromolecular film in none of the methods compared (Fig. 3b).

4. Discussion

Quaternary ammonium coatings possess the unique feature of killing bacteria upon contact, which offers a promising alternative for antibiotic-based approaches, particularly considering the rise in the number of antibiotic resistant strains and species developing [34,38]. Unfortunately, no ubiquitously accepted method exists to properly evaluate the efficacy of contact-killing materials or coatings. In addition, results may be obscured by the fact that for many antibacterial materials reported on in the literature, it is not clear whether effects are due to leaching of residual antimicrobial compounds [17,20,25,39,40] ('release-killing') from a contact-killing material or due to contact-killing itself. Here we have compared five different methods (see Table 1) with respect to their virtues for evaluating the contact-killing efficacy of an established, non-leaching contact-killing coating of quaternary ammonium compounds covalently bound in a UDMA/GDMA resin against five different bacterial strains.

In Table 1 we give an overview of the advantages and disadvantages of each used contact-killing method, as perceived based on the current comparison and discussed below. Two out of the five methods evaluated demonstrated clear contact-killing efficacy against Gram-positive bacteria (Petrifilm® and JIS Z 2801). Efficacies against a Gram-negative bacterium were slightly less compared to Gram-positive strains, presumably due to the possession of a double membrane by Gram-negative bacteria [41–43]. Bacterial spraying and ASTM E2149-13a yielded no indications of contact-killing, while the modified JIS method yielded growth on a contact-killing UDMA/GDMA/QA_{C12} surface, although less than on a control UDMA/GDMA material without quaternary ammonium incorporated. Percentage bacterial contact-killing ranked as follow in the different methods: JIS Z 2801 = Petrfilm® > modified JIS > bacterial spray = ASTM E2149-13a. Differences obtained between JIS Z 2801 and Petrfilm® are not significant and the methods are in fact highly comparable. In both methods bacteria are

Fig. 3. Bacterial contact-killing efficacy (%) achieved in the various methods on UDMA/GDMA/QA_{C12} in absence and presence of an adsorbed macromolecular film. Percentage efficacy is defined as the log reduction achieved divided by the maximally possible log reduction. Error bars indicate the SD over triplicate experiments with separately cultured bacteria. (a) Bacterial contact-killing efficacy (%) achieved in the various methods for five bacterial strains in absence of an adsorbed macromolecular film. (b) Bacterial contact killing efficacy (%) achieved in the various methods for two bacterial strains, for uncoated and UDMA/GDMA/QA_{C12} with a macromolecular conditioning film. The samples were coated overnight with 10% fetal calf serum (dotted columns) for *S. epidermidis* ATCC12228 or saliva (shaded columns) for *S. mutans* NS.

contacted with a contact-killing surface within a very small fluid volume, ensuring contact (see also Table 1). However, the Petrifilm® method may be considered slightly more convenient than JIS Z 2801 because surviving bacteria are grown into countable colonies during contacting. In JIS Z 2801 bacteria have to be dislodged by sonication after adhering strongly to the sample through electrostatic attraction [39], which not only constitutes an additional step with respect to the Petrifilm® method, but possibly also explaining the slightly higher contact-killing efficacy in JIS Z 2801

as the forceful sonication required may yield additional killing. The small fluid volumes in which bacteria and contact-killing surfaces are brought together, also constitute a possible danger of these two methods, as extremely small amounts of antimicrobial leachables may easily cause the build-up of a high concentration of antimicrobial compounds to interfere with contact-killing. Therefore it is needed to use JIS Z 2801 and Petrifilm® in combination with an agar zone of inhibition assay to ascertain that there is no release of antimicrobial compounds with demonstrable biological effects.

The presence of a balanced amount of nutrients in the Petrifilm® is considered as an advantage, depending on the application of the coatings aimed for.

The modified JIS method [31] was developed in order to provide an opportunity to the bacteria to grow during adhesion to a contact-killing surface, which makes it similar in principle to the Petrifilm® method (Table 1). Yet in the modified JIS method, the growth opportunities are too much and easily overshadow contact-killing by the surface: the contact-killing efficacies for two Gram-positive bacterial strains (*E. faecalis* and *S. mutans*) are clearly smaller compared to results obtained in the Petrifilm® and JIS Z 2801 method, whereas for Gram-negative *E. coli* in the modified JIS method, survivors were able to grow to the extent that “negative killing” was measured. Accordingly, the modified JIS method is only useful when comparing the growth observed on a contact-killing surface with the growth on a non-contact-killing, control surface such as the UDMA/GDMA sample in the current study. Although advocated as an advantage of the modified JIS method that it can be applied in the presence of a pre-adsorbed proteinaceous conditioning film, this advantage is not exclusive to the modified JIS method and can be equally applied in all other methods (see also Fig. 3b).

The bacterial spray method is used in several papers [13,21,32] and often demonstrated 100% contact-killing amongst others against *S. aureus* and *E. coli* strains, but yielded no worthwhile contact-killing in the current comparison of methods. Self-admitted also by the designers of the bacterial spray method [32], determination of the challenge number of bacteria that actually come into contact with a coating is hard to establish and can easily be over- or underestimated and cause erroneous contact-killing efficacies. Yet, for air-borne bacterial contamination of surfaces, the spray method remains the only one available, although alternatively, air-borne contaminated surfaces might be further analyzed in the Petrifilm® assay with greater accuracy. The ASTM E2149-13a method [29] only yielded minor bacterial contact-killing efficacy, although contact-killing efficacies against *E. coli* after 30 min between 50% and 100% have been reported for different contact-killing, cationic coatings [44,45]. Experiments carried out as a function of agitation time in this study demonstrated that killing efficacies did not further increase with time (Suppl. Fig. 1). The advantage of the method is that samples with a variety of shapes and sizes can be used, but the method fails to control the challenge number of bacteria that actually come into contact with a surface. Also bacterial enumeration is indirect, since aliquots are taken from the suspension for CFU analysis, instead of measuring directly on the contact-killing surface. Therewith it is impossible to distinguish between killed and adhering bacteria that are still alive. Originally, ASTM E2149 was designed for testing the antimicrobial activity, i.e. contact-killing of non-leaching, antimicrobial surfaces under dynamic contact conditions. The current comparison of methods to evaluate bacterial contact-killing by antimicrobial surfaces points out that even after several revisions [29] ASTM E2149-13a remains unsuitable to evaluate bacterial contact-killing and will likely be more suitable to evaluate antimicrobial-release coatings rather than contact-killing ones. In line with the conclusions of this paper, the last revision of ASTM2149-13a emphasizes that the test cannot exclude leaching, and recommends additional assays to exclude “release-killing” by leaching of antimicrobials from a sample.

5. Conclusions

This study reveals that depending on the method used, different efficacies can be obtained in the evaluation of bacterial contact-killing surfaces. We conclude that the Petrifilm® and JIS Z 2801

are preferable methods, with Petrifilm® being most convenient and possibly more reliable due to a balanced amount of nutrients. Like all others, Petrifilm® and JIS Z 2801 need a complementary assay to exclude killing due to release of antimicrobial compounds, because even a small release of an antimicrobial compound can have a large influence on bacterial killing. The modified JIS method is acceptable, but does not contain balanced amount of nutrients compared to the Petrifilm® and JIS Z 2801 method. Therefore the modified JIS method should only be used with respect to a non-contact killing control. ASTM E2149-13a and bacterial spray methods are not reliable, the main reason being the lack of control over the applied bacterial challenge and actual contact of bacteria with the surface.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actbio.2017.06.042>.

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