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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.

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 quaternarized 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 Kα 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 $1000 \times 250\ \mu m^2$ 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

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

in which Abs_{501} is the UV absorption at 501 nm, ε_{501} is the extinction coefficient (77 $mM^{-1}\ cm^{-1}$ 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:

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