Charge properties and bacterial contact-killing of hyperbranched polyurea-polyethyleneimine coatings with various degrees of alkylation

Steven Roest a, Henny C. van der Mei a,∗, Ton J.A. Loontjens b, Henk J. Busscher a

a University of Groningen, University Medical Center Groningen, Department of Biomedical Engineering, AntoniusDeusinglaan 1, 9713 AV Groningen, The Netherlands
b University of Groningen, Zernike Institute for Advanced Materials, Department of Polymer Chemistry, Nijenborgh 4, 9747 AG Groningen, The Netherlands

A R T I C L E   I N F O

Article history:
Received 26 March 2015
Received in revised form 6 August 2015
Accepted 11 August 2015
Available online 13 August 2015

Keywords:
Anti-bacterial
Polyurea
Polyethyleneimine
Quaternary ammonium
X-ray photoelectron spectroscopy
Fluorescein

A B S T R A C T

Coatings of immobilized-quaternary-ammonium-ions (QUAT) uniquely kill adhering bacteria upon contact. QUAT-coatings require a minimal cationic-charge surface density for effective contact-killing of adhering bacteria of around 1014 cm−2. Quaternization of nitrogen is generally achieved through alkylation. Here, we investigate the contribution of additional alkylation with methyl-iodide to the cationic-charge density of hexyl-bromide alkylated, hyperbranched polyurea-polyethyleneimine coatings measuring charge density with fluorescein staining. X-ray-photoelectron-spectroscopy was used to determine the at.% alkylated-nitrogen. Also streaming potentials, water contact-angles and bacterial contact-killing were measured. Cationic-charge density increased with methyl-iodide alkylation times up to 18 h, accompanied by an increase in the at.% alkylated-nitrogen. Zeta-potentials became more negative upon alkylation as a result of shielding of cationic-charges by hydrophobic alkyl-chains. Contact-killing of Gram-positive Staphylococci only occurred when the cationic-charge density exceeded 1016 cm−2 and was carried by alkylated-nitrogen (electron-binding energy 401.3 eV). Gram-negative Escherichia coli was not killed upon contact with the coatings. There with this study reveals that cationic-charge density is neither appropriate nor sufficient to determine the ability of QUAT-coatings to kill adhering bacteria. Alternatively, the at.% of alkylated-nitrogen at 401.3 eV is proposed, as it reflects both cationic-charge and its carrier. The at.% N401.3 eV should be above 0.45 at.% for Gram-positive bacterial contact-killing.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Hyperbranched polymers are dendritic molecules with unique chemical and physical properties that have great technological potential as novel adhesives [1–4], lubricants [5], photolithographic masks [6], polymer surfactants [7,8], polymer compatibilizers [7], protein-resistant [3,4] and bacteria-killing materials [9–11]. Via “grafting on”, modified hyperbranched poly(acrylic acid) [12–15], polyethyleneimine (PEI) [16] and poly(vinyl-N-pyridinium) have been coupled to a variety of materials [17]. “Grafting on” however, bears as a disadvantage that, due to steric hindrance, the maximum grafting density is limited. This disadvantage is circumvented with “grafting from”, that often starts by pre-treatment of surfaces, e.g. with a coupling agent. A very suitable coupling agent to graft polymers “from” hydroxyl bearing surfaces is 3-aminopropytriethoxysilane (APTS). Moreover, APTS can be provided with functional groups, prior to coupling to a material surface. Via this method, an even broader set of reactions is possible and e.g. robust hyperbranched polyurea-PEI coatings have been created via this route [9,18].

Coatings comprising immobilized cationic species, e.g. quaternary ammonium compounds (QUATs), have the unique ability to kill adhering bacteria upon contact [11,19,20], therewith preventing them from growing into a biofilm and switching on their protective mechanisms, that make them difficult to eradicate by antimicrobials [21]. Interestingly, such cationic coatings can kill bacteria, that are resistant to the same cation in solution [19], demonstrating that the working mechanism of coatings consisting...
of immobilized cations is different from the one of cations in solution. It has been suggested [10] that strong electrostatic, attractive forces between immobilized cations and anionic lipids in bacterial cell membranes cause removal of membrane lipids destroying the cytoplasm membrane, leading to cell death. Evidence in support of this hypothesis has recently been provided by atomic force microscopic measurements of the adhesion forces acting on bacteria by cationic polyurea-PEI coatings, showing lethally strong adhesion forces [9]. These forces are nearly ten-fold higher than on surfaces on which adhering bacteria thrive and form a biofilm.

Bacterial cell surfaces are negatively charged under natural conditions [22]. The development of attractive forces between adhering bacteria and immobilized cationic coatings, being sufficiently strong to eliminate anionic membrane lipids, requires a minimum cationic charge density. Methods to directly measure this surface charge density are scarce. Fluorescein staining followed by UV/VIS spectroscopy is the most commonly applied method to this end. Using fluorescein staining, a minimal charge density of $10^{14}$ quaternary ammonium groups per cm$^2$ has been suggested for effective contact-killing of adhering bacteria [23,24].

Recently we prepared contact-killing hyperbranched coatings by grafting PEI onto a hyperbranched polyurea coating [9]. Subsequently, the PEI was alkylated with hexyl bromide and methyl iodide. The second alklylation step using methyl iodide was conducted to enhance the cationic charge density of the coating to enhance bacterial contact-killing. However, it is not clear to what extent the additional alklylation steps actually contribute to the cationic charge in the coating and bacterial contact-killing. Therefore, the aim of this paper is to determine the influence of the type and amount of cationic species in hyperbranched polyurea-PEI coatings on bacterial contact-killing [9]. Cationic charge density is measured using fluorescein staining, while the presence of alkylated nitrogen is demonstrated using X-ray photoelectron spectroscopy (XPS). Furthermore, streaming potentials and water contact angles are measured on the various coatings and cationic charge density is related with the contact-killing of three Gram-positive staphylococcal strains and one Gram-negative Escherichia coli strain, which are frequently found in biomaterial implant-associated infections [25].

2. Materials and methods

2.1. Materials

Glass slides (2.6 cm × 7.6 cm) were obtained from Waldemar Knittel® (Braunschweig, Germany). Glass slides were cut to other dimensions when experimental conditions required such. Bis(hexamethylenetetramine), (3-aminopropyl)triethoxysilane, polyethyleneimine (750 kDa, 50 wt.% in water), methyl iodide, 2-methyl-2-butanol, fluorescein disodium salt, hexyl bromide and cetyltrimethylammonium chloride were all purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Potassium hydroxide and dimethylformamide were purchased from Acros organic (Geel, Belgium). Sulfuric acid, hydrogen peroxide and 100% ethanol were obtained from Merck (Amsterdam, The Netherlands). Methanol and toluene were obtained from Lab-Scan (Gliwice, Poland). Carbonyl biscalprolactam (>99%) was kindly provided by DSM innovation center, ALLINCO® (Urmond, The Netherlands). All chemicals were used as received.

2.2. Preparation and alkylation of hyperbranchedpolyurea-PEI coatings

AB$_2$ monomers, consisting of a secondary amino (A) group and two blocked isocyanate (B) groups separated by hexyl spacers

and the corresponding hyperbranched polymer coatings were essentially prepared as described by Asri et al. [9]. Note that whereas Asri et al. [9] used AB$_2$ monomers directly, we allowed the AB$_2$ monomers to polymerize in dimethylformamide solution for 40 min at 145 °C. Glass slides were activated with piranha treatment and subsequently functionalized with 2-oxo-3-[3-(triethoxysilyl)propy]azine-1-carboxamide as a coupling agent. The pure hyperbranched polymer was obtained by precipitation in cold water and dried under reduced pressure at 40 °C. The functionalized glass slides were submerged in a solution of 5 wt.% hyperbranched polymer and subsequently spinneled at 2000 rpm for 60 s. After annealing, non-anchored polymers were removed by a three-step extraction. First, the functionalized glass slides were sonicated in ethanol at room temperature (RT) for 20 min, followed by overnight immersion in dimethylformamide at 115 °C, sonication in ethanol at RT for 20 min and finally dried under nitrogen. Solutions of PEI (0.10 wt.%, 10 wt.%, 15.7 wt.% or 20 wt.%) in methanol (800 µl) were dropped on the hyperbranched coating and spin coated. The coating reactions were carried out on an aluminum plate heated to 125 °C for 52 h under nitrogen, followed by intermittent sonication in methanol at RT for 2 × 45 min in fresh solvent to extract unreacted components. Next, the glass slides with polyurea-PEI functionalized hyperbranched coatings were immersed in 150 ml hexyl bromide and heated under nitrogen at 90 °C overnight for alklylation. A suspension of 0.6 g potassium hydroxide powder in 50 ml tert-amyl alcohol was added. The reaction was continued for another 3 h at 90 °C. Afterwards, coatings were three times sonicated in methanol for 20 min at RT and dried under nitrogen. A second alklylation step was done in a round bottom flask fitted with a reflux condenser. The coatings were immersed in a solution of 20 ml methyl iodide in 150 ml tert-amyl alcohol. Alklylation was carried out at 42 °C for time periods between 0 and 18 h. Subsequently, samples were sonicated in 100 ml methanol for 20 min at RT and followed by extraction in methanol at 65 °C for 1 day and another sonication in methanol for 20 min at RT. Finally, the QUAT coated slides were dried and stored under nitrogen. In addition, experiments were carried out in absence of any alklylation for coatings prepared with 0.10 wt.%, 10 wt.%, 15 wt.%, 17.5 wt.% or 20 wt.% PEI only. See Fig. 1 for a schematic drawing of the hyperbranched polyurea coating.

2.3. Cationic charge density using fluorescein staining

The cationic charge density of the coatings was determined using fluorescein staining. To this end, coated glass slides were immersed at RT in 15 ml 1 wt.% fluorescein (disodium salt) solution in demineralized water for 10 min, washed four times with 50 ml water, followed by sonication in 50 ml water for 5 min at RT to remove any dye not complexed with cationic charges, as due e.g. to alkylated or protonated nitrogen species. Next, the samples were placed in 10 ml of a 0.1 wt.% cetyltrimethylammonium chloride solution in demineralized water and sonicated for 10 min at RT to desorb complexed fluorescein dye. Subsequently, 10 v/v% of 100 mM phosphate buffer, pH 8, was added to a total volume of 11 ml and UV/VIS measurements (Spectra max M2 UV/VIS spectrophotometer) carried out at 501 nm to yield the concentration of fluorescein dye in the extraction solution [Dye] in M according to

$$[\text{Dye}] = \frac{\text{Abs}_{501}}{s_{501}} \times L$$

in which Abs$_{501}$ is the UV absorption at 501 nm, $s_{501}$ is the extinction coefficient at 501 nm (77 mM$^{-1}$ cm$^{-1}$ for fluorescein) and L is the length of a polystyrene cuvette (1 cm) traversed by the UV-light beam.
Next, the cationic charge density per cm² glass slide was calculated using Eq. (2) [26]

\[
\text{Charge density} = \frac{[\text{Dye}] \times V \times N/A}{A}
\]

in which \( V \) is the volume of the extraction solution (11 ml), \( N \) is Avogadro’s number (6.023 \times 10^{23}) and \( A \) is the surface area of the glass slide (6.76 cm²).

### 2.4. Zeta potential

For a solid surface in contact with a liquid, streaming potentials \( \Delta E_{\text{str}} \) arise from a forced flow of the liquid under the influence of a pressure \( \Delta p \), that depend on the zeta potential \( \zeta \) at the solid-liquid interface according to

\[
\frac{\Delta E_{\text{str}}}{\Delta p} = \frac{\varepsilon \varepsilon_0 \eta \kappa_p}{\zeta}
\]

in which \( \varepsilon \varepsilon_0 \) is the dielectric permittivity, \( \eta \) the viscosity and \( \kappa_p \) the specific conductivity of the liquid. The pressure dependence of the streaming potentials was measured in a parallel plate flow chamber. The walls of the flow chamber were made of QUAT coated glass slides (7.6 cm × 2.6 cm), separated by a 0.2 mm Teflon gasket, while two rectangular platinum electrodes (5.0 mm × 25.0 mm) were located at both ends of the parallel plate flow chamber. Streaming potentials were measured in 1 mM KNO₃ at ten different pressures ranging from 50 to 450 mbar. Each pressure was applied for 10 s in both directions and zeta potentials calculated using Eq. (3).

### 2.5. X-ray photoelectron spectroscopy

XPS was performed using an S-Probe spectrometer (Surface Science Instruments, Mountain View, CA, USA) equipped with an aluminum anode (10 kV, 22 mA) and a quartz monochromator on 2.6 × 2.6 cm glass slides. The direction of the photoelectron collection angle was 55 degrees with the normal to the sample, implying measurement of composition over the outermost 1-2 nm of the coating, i.e. the approximate electron mean free path length in polymers. The electron flood gun was set at 10 eV. A survey scan over a binding energy range of 1100 eV was made with a 1000 × 250 μm spot and a pass energy of 150 eV. Binding energies were determined by setting the binding energy of the C1s binding energy peak (carbon bound to carbon) at 284.8 eV. Detailed scans of the N1s binding energy peaks over a binding energy range of 20 eV were made using a pass energy of 50 eV. The N1s peak was subsequently decomposed in two fractions at 399.3 and 401.3 eV. The occurrence of a peak at 401.3 eV was interpreted as due to alkylated nitrogen species and expressed in at.% by multiplication of the peak fraction with the total at.% nitrogen.

### 2.6. Contact angle measurements

Advancing type water contact angles on the functionalized glass slides were measured at RT with ultrapure water using a contour monitor with gray-value thresholding. One μl droplets were applied with a Hamilton microsyringe and contact angles were measured after 5 s. All contact angles reported are averages of measurements on three different spots on three separately prepared coatings.

#### 2.7. Bacterial contact-killing

Gram-positive *S. epidermidis* ATCC 12228, *S. epidermidis* 252, *Staphylococcus aureus* 5298 and Gram-negative *Escherichia coli* ATCC 15597 were used in this study. The strains were first streaked on blood agar plates from frozen stock solutions (7 v/v% DMSO) and grown overnight at 37 °C. One colony was inoculated in 10 ml tryptone soya broth (TSB, Oxoid, Basingstoke, UK) for the staphylococcal strains and brain heart infusion broth (BHI, Oxoid) for *E. coli* and incubated at 37 °C for 24 h. Ten mL of these cultures were used to inoculate a main culture of 200 ml TSB or BHI, which was incubated for 16 h at 37 °C. Bacteria were harvested by centrifugation for 5 min at 6500 g and 10 °C and subsequently washed two times with 10 mM potassium-phosphate buffer, pH 7.0. Bacterial challenge concentrations of 2 × 10⁴ and 2 × 10⁵ colony forming units (CFU)/ml were used for *S. epidermidis* ATCC 12228, while for the other three strains only the highest concentration was used to evaluate the contact-killing of the coating employing a Petrofilm® Aerobic Count plate system (3 M Microbiology, St. Paul, MN, USA). The Petrofilm® system 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 films. The bottom film containing the gelling-agent was first swelled with 1 mL sterile demineralized water for 40 min and transferred to the transparent top film before usage. Next, 40 μl of a bacterial suspension was placed on bare as well as on functionalized glass slides (2.6 × 2.6 cm), placed in the Petrofilm® system and subsequently the Petrofilm® system was closed. The bacterial suspension spread over the entire surface area of the samples, enabling calculation of the bacterial challenge per cm² from the dimensions of the samples and the bacterial concentration in suspension. Petrofilms® were incubated at 37 °C for 48 h after which the numbers of CFU were counted. As a control, 40 μl of the bacterial suspension was inoculated in a Petrofilm® system without a sample in between.

### 3. Results

First, surface charge densities of the hyperbranched polyurea-PEI coatings alkylated with hexyl bromide were determined as a function of the time during which additional alkylation with methyl iodide was carried out using fluoroscene staining (Fig. 2). Prior to alkylation with methyl iodide (zero time points in Fig. 2), cationic charge densities increased with increasing wt.% of PEI, while also increasing alkylation times with methyl iodide yielded an increase in cationic charge density up to at least 18 h.

Next, the wt.% of nitrogen species at 401.3 eV and 399.3 eV as measured using XPS was derived from a decomposition of N1s binding energy peaks (see Fig. 3 for an example) and the total wt.% nitrogen. Interestingly, the cationic charge density derived from
fluorescein staining relates nearly linear with the at.% of nitrogen species at 401.3 eV (Fig. 4).

Zeta potentials reflect the electrostatic potential around an interface and were found to become more negative upon increasing the methyl iodide alkylation time (Fig. 5). For the lowest wt.% PEI in solution, zeta potentials became more positive with increasing alkylation time as expected upon alkylation (see inset to Fig. 5), while for all other wt.% PEI included zeta potential became more negative upon alkylation. Most negative values were found for coatings prepared from solutions with 15 wt.% PEI in solution, while both higher and lower wt.% of PEI in solution yielded zeta potentials closer to zero.

Water contact angles on the different hyperbranched PEI coatings hovered around 90 degrees and did not vary in a systematic way neither with the wt.% PEI in solution nor with methyl iodide alkylation time (see Fig. 6). For hyperbranched PEI coatings
prepared from 0.1 wt.% and 15 wt.% PEI in solution, no influence of alklylation time was observed, but for coatings prepared from 10, 17.5 and 20 wt.% PEI in solution a small, but systematic decrease in water contact angle is seen upon methyl iodide alklylation for 18 h, as compared to absence of methyl iodide alklylation.

The efficacy of the hyperbranched polyurea-PEI coatings to kill Gram-positive *S. epidermidis* ATCC 12228 upon contact as a function of the cationic charge density in the coatings, was first evaluated at a low bacterial challenge, as presented in Fig. 7a. At the higher bacterial challenge, two additional staphylococcal strains were included. A Gram-negative *E. coli* strain was not killed upon contact with the coatings. For the lowest bacterial challenge, the 100% level represents 2 log-units reduction in CFU, while for the higher challenge the 100% level indicates 4 log-units reduction. At the lower bacterial challenge for *S. epidermidis* ATCC 12228 (Fig. 7a), contact-killing remains low and started to increase gradually around a cationic charge density of $10^{16}$ cm$^{-2}$ to the 100% level. A similar pattern is observed at the higher challenge (Fig. 7b) for all three staphylococcal strains. The main difference in pattern between the two bacterial challenges for *S. epidermidis* ATCC 12228 is seen for coatings prepared from solutions with the higher wt.% of PEI, showing low percentage killing above $10^{16}$ cm$^{-2}$ at the lower bacterial challenge. For the higher challenge however, all coatings prepared from solutions with more than 0.1 wt.% PEI (except for the 10 wt.% PEI solution with 0 h methyl iodine alklylation), high percentages of staphylococcal contact-killing above 65% were observed.

Since our polyurea-PEI coatings not only contain positive charges due to alklylation but also due to protonation of nitrogen in PEI, we investigated the physico-chemical properties and contact-killing ability of PEI coatings for *S. epidermidis* ATCC 12228 prepared from solutions with different wt.% PEI in absence of any alklylation (Table 1). The cationic charge density measured using fluorescein staining increased with increasing wt.% PEI in solution and was within the range of the alklylated PEI coatings (compare Fig. 2). Yet, using XPS, the at.% of nitrogen species at 401.3 eV was found close to zero regardless of the wt.% PEI in solution during the coating preparation. Zeta potentials were all negative as in case of alklylated PEI coatings (compare Fig. 5), but only within the range of the alklylated coatings when prepared from a solution with 0.1 wt.% PEI. When prepared from solutions with a higher wt.% PEI, zeta potentials of PEI coatings prepared without alklylation were always less negative than when prepared with alklylation. Alklylation not only had a significant effect on the occurrence of nitrogen species at 401.3 eV, but also on the hydrophobic nature of the coatings. Alklylation with hexyl bromide increased the water contact angles of the coatings from about 40–90 degrees (compare Table 1 and Fig. 6). Staphylococcal contact-killing of coatings prepared without any alklylation was absent for the high bacterial

---

**Fig. 5.** The cationic charge density in polyurea-PEI coatings, prepared from PEI solutions with various wt.% PEI alkylated with hexyl bromide and methyl iodide for various alklylation times (derived from fluorescein staining, see Fig. 2), as a function of the zeta potentials measured in 1 mM KNO$_3$. For clarity, data for 0.1 wt.% PEI in solution are shown in the inset. Arrows point in the direction of increasing methyl iodidealklylation time up to 18 h and data represent averages over 3 independent measurements with separately prepared coatings.

**Fig. 6.** Water contact angles on polyurea-PEI coatings, prepared from PEI solutions with different wt.% PEI alkylated with hexyl bromide and methyl iodide for various methylation times. Error bars represent the standard deviations over measurements on three different spots on at least three separately prepared coatings.
challenge, even though the cationic charge density was above the
threshold density of $1 \times 10^{16}$ cm$^{-2}$ observed for alkylated coatings
(compare Table 1 and Fig. 7b). For the lower staphylococcal challenge, contact-killing was only observed for PEI coatings prepared
from solution with 17.5 and 20 wt.% PEI, although two-fold less than observed on similar coatings after alkylation (compare Table 1
and Fig. 7a).

4. Discussion

In this paper we aim to gain more insight in the cationic charge
carriers in hyperbranched polyurea-PEI coatings that facilitate
bacterial contact-killing. For that purpose a two-step alkylation process was performed. The first alkylation step was conducted
with hexyl bromide to introduce hydrophobicity, which is indispensible, beside a high charge density, to acquire contact-killing
properties. A hexyl group was chosen because it was previously shown to perform well [9]. The cationic charge was further
increased stepwise by alkylation with methyl iodide during various
reaction times. Cationic charge density was determined by fluo-
rescein staining as the most commonly applied method to this end
and it has been shown by numerous groups that a cationic charge
density of more than $1 \times 10^{14}$ cm$^{-2}$ as determined by fluorescein
staining, is required for effective bacterial contact-killing [23,24].

Fig. 7. Percentage log-reduction in the number of staphylococcal CFUs at two staphylococcal challenges (88 and 8800 CFU/cm$^2$) as a function of the cationic charge density in polyurea-PEI coatings, prepared from PEI solutions with various wt.% PEI alkylated with hexyl bromide and methyl iodide for various times (see also Fig. 2). Data represent averages over 3 independent measurements with separately prepared coatings and bacterial cultures. 100% levels represent the maximal log-reductions in staphylococcal counts possible considering the bacterial challenge applied. (a) A low staphylococcal challenge ($S$. epidermidis ATCC 12228) of 88 CFU/cm$^2$. (b) A high bacterial challenge of 8800 CFU/cm$^2$ for all three staphylococcal strains.

Table 1

Physical-chemical surface properties of coatings prepared from solutions with various wt.% PEI in solution without any alkylation and percentage log-reduction of $S$. epidermidis ATCC 12228 at challenges of 88 and 8800 CFU/cm$^2$ (100% levels represent the maximal log-reductions in staphylococcal counts possible considering the bacterial challenge applied). ± signs represent the standard deviations over 2 measurements with independently prepared coatings.

<table>
<thead>
<tr>
<th>PEI (wt.%)</th>
<th>Cationic charge density ($\times 10^{10}$ cm$^{-2}$)</th>
<th>at. % at 401.3 eV</th>
<th>Zeta potential (mV)</th>
<th>Water Contact angle (degrees)</th>
<th>Log-reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.0 ± 0.0</td>
<td>0.23 ± 0.03</td>
<td>$-5.4 \pm 0.9$</td>
<td>52 ± 4</td>
<td>0 tmtc</td>
</tr>
<tr>
<td>10</td>
<td>3.0 ± 0.6</td>
<td>0.00 ± 0.00</td>
<td>$-2.4 \pm 0.4$</td>
<td>35 ± 2</td>
<td>0 tmtc</td>
</tr>
<tr>
<td>15</td>
<td>3.0 ± 0.0</td>
<td>0.21 ± 0.14</td>
<td>$-1.7 \pm 0.9$</td>
<td>41 ± 5</td>
<td>0 tmtc</td>
</tr>
<tr>
<td>17.5</td>
<td>4.1 ± 0.1</td>
<td>0.00 ± 0.00</td>
<td>$-0.4 \pm 0.1$</td>
<td>35 ± 2</td>
<td>25 tmtc</td>
</tr>
<tr>
<td>20</td>
<td>4.1 ± 0.1</td>
<td>0.00 ± 0.00</td>
<td>$-1.2 \pm 0.2$</td>
<td>33 ± 3</td>
<td>42 tmtc</td>
</tr>
</tbody>
</table>

* Measured by fluorescein method.
* Measured by XPS.
* tmtc = too many to count.
during 24 h, confirming that the biocidal efficacy is solely due to contact-killing and not to leaching [9]. Absence of leaching would also explain why our coatings do not kill Gram-negative E. coli. In our experience, Gram-negative bacterial strains are not killed by cationic coatings due to their double lipid membrane and only dissolved QUAT kill Gram-negative strains (unpublished). Very few studies [11] on bacterial-contact killing by QUAT coatings have established absence of leaching of antimicrobial compounds in the same rigorous way as we have done, which would explain our observation of Gram-positive bacterial killing at a 100-fold higher threshold for the cationic charge density than generally reported in the literature. Moreover, bacterial contact-killing is an interfacial phenomenon [9,11,26] requiring immobilized cations to be present at the outermost surface. Fluorescein staining is not a very surface-sensitive method and considering the thickness of our hyperbranched coatings with respect to the mostly mono-molecular coatings prepared in the literature [27], it is likely that fluorescein staining also probes cationic charge carriers in the depth of our coatings, which would provide an alternative explanation as to why we find a 100-fold higher threshold for the cationic charge density required for bacterial contact-killing.

However, we also found another discrepancy with current literature: Polyurea-PEI coatings prepared without any alkylation possessed similar cationic charge densities above reported thresholds in the literature (and confirmed in this study) for alkylated polyurea-PEI coatings, but demonstrated no significant staphyloccal contact-killing. Thus it must be concluded that cationic charge density as determined by fluorescein staining is not a sufficient indicator of the bacterial contact-killing ability of QUAT coated surfaces, but also the type of charge carrier matters. The fluorescein staining is performed in an aqueous environment and as a result, it will also measure the cationic charge arising from protonated amino groups of PEI itself. This conclusion is confirmed by XPS-analyses. XPS, contrary to fluorescein staining, is done in a high vacuum environment and amino groups, protonated in water during fluorescein staining and absorbance measurements are transformed to uncharged amino groups upon removal of water. Accordingly, XPS detects virtually no nitrogen at 401.3 eV in absence of alkylation (see Table 1), despite the measurement of identical cationic charge densities as found after alkylation. Moreover, this confirms that nitrogen species with an electron binding energy at 401.3 eV in our polyurea-PEI coatings can be attributed to alkylated nitrogen species as the required cationic charge carrier for bacterial contact-killing.

In streaming potential measurements, the zeta potential decreased with increasing alkylation time. This is a seemingly counter-intuitive trend, since the cationic surface charge density increases (Fig. 2). We have two potential explanations for this counter-intuitive trend. Firstly, positively charged alkylated ammonium groups are likely to become shielded by the alky groups introduced by (additional) alkylation. This explanation is supported by the fact that the alkylated coatings have a much higher water contact angles by approximately 40–50 degrees than non-alkylated ones. As a second explanation, more negatively charged counter ions will adsorb to the surface due to the higher cationic charge density [28], although these may adsorb quite reversibly as they do not impede bacterial contact-killing.

Staphylococcal contact-killing was evaluated at two bacterial challenges, differing over a factor of 100. The lower challenge allows killing of maximally 88 CFU, which makes it statistically less reliable than the higher challenge allowing killing of maximally 8800 CFU. Yet it is of interest to note that there are often surviving bacteria after a challenge with 88 bacteria cm⁻². Speculatively, these represent bacteria that have found heterogeneities in the coating that allow them to evade contact-killing and survive. The existence of chemical heterogeneity in the coatings was confirmed by the presence of a hysteresis in water contact angles, amounting 27 degrees on average. Clearly, the survival of such a small number of staphylococci does not imply the survival of higher numbers of staphylococci at a higher challenge and highly effective contact-killing at a challenge of 8800 bacteria cm⁻² close to the 100% level was observed. This puts emphasis on the exact, quantitative determination of the bacterial challenge. The Petrofilm assay as applied here is ideal to that end, because high challenges can be quantified accurately from the volume of the bacterial suspension used and the concentration of viable bacteria therein. In other methods, such as spray-coating of bacteria onto a surface, it remains self-admitted [29] impossible to obtain a reliable estimate of high bacterial challenges.

Bacterial contact-killing is an interfacial phenomenon controlled by the cationic charge density and its carrier type at the outermost surface of a material. Results of physico-chemical analyses of PEI coatings after alkylation and their bacterial contact-killing efficacy demonstrate a threshold cationic charge density as obtained from fluorescein staining for bacterial contact-killing to occur. However in absence of alkylation, fluorescein staining shows similar cationic charge densities but such coatings lack the potential for contact-killing, demonstrating that fluorescein staining is not an appropriate method to determine the interfacial cationic charge density, a more appropriate method being XPS possessing a much higher surface-sensitivity of only 1–2 nm.

5. Conclusions

This study reveals first of all that cationic charge density is not an appropriate measure to determine the ability of QUATs coatings to kill adhering bacteria upon contact. Cationic charge density is usually measured using fluorescein staining, which not only penetrates into a coating, but moreover does not distinguish between the type of charge carrier. We here demonstrate that only the cationic charge carried by alkylated nitrogen species contributes to bacterial contact-killing and conclude from our data that a threshold of minimally 0.45 at.% of alkylated nitrogen at a binding energy of around 401.3 eV is required for Gram-positive bacterial contact-killing.

Disclosures

HJB is also director of a consulting company, SASA BV (GN Schutertlaan 4, 9797 PC Theisinge, The Netherlands). The authors declare no potential conflicts of interest with respect to authorship and/or publication of this article. Opinions and assertions contained herein are those of the authors and are not construed as necessarily representing views of the funding organizations or their respective employers.

Acknowledgements

This project was funded by the UMCG.

References


