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

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ABSTRACT

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 $10^{14}$ 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 cationiccharges by hydrophobic alkyl-chains. Contact-killing of Gram-positive *Staphylococci* only occurred when the cationic-charge density exceeded $10^{16}$ 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.% $N_{401.3\,eV}$ should be above 0.45 at.% for Gram-positive bacterial contact-killing.



## 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], polyethylenimine (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 pretreatment of surfaces, e.g. with a coupling agent. A very suitable coupling agent to graft polymers "from" hydroxyl bearing surfaces is 3-aminopropyltriethoxysilane (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

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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 alkylation 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 alkylation 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. Bishexamethylenetriamine, (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 biscaprolactam (>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-N(3-triethoxysilyl)propyl)azepane-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 spinned 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 wt.%, 17.5 wt.% or 20 wt.%) in methanol (800 μl) were dropped on the hyperbranched coating and spin coated. The grafting 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 alkylation. 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 alkylation 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. Alkylation 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 alkylation 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

$$[\mathrm{Dye}] = \frac{\mathrm{Abs}_{501}}{\varepsilon_{501} \mathrm{xL}} \quad (1)$$

in which $\mathrm{Abs}_{501}$ is the UV absorption at 501 nm, $\varepsilon_{501}$ 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.