Penetration and Accumulation of Dendrons with Different Peripheral Composition in *Pseudomonas aeruginosa* Biofilms

René T. Rozenbaum, Oliver C. J. Andrén, Henny C. van der Mei, Willem Woudstra, Henk J. Busscher, Michael Malkoch, and Prashant K. Sharma

ABSTRACT: Multidrug resistant bacterial infections threaten to become the number one cause of death by the year 2050. Development of antimicrobial dendritic polymers is considered promising as an alternative infection control strategy. For antimicrobial dendritic polymers to effectively kill bacteria residing in infectious biofilms, they have to penetrate and accumulate deep into biofilms. Biofilms are often recalcitrant to antimicrobial penetration and accumulation. Therefore, this work aims to determine the role of compact dendrons with different peripheral composition in their penetration into *Pseudomonas aeruginosa* biofilms. Red fluorescently labeled dendrons with pH-responsive NH$_3^+$ peripheral groups initially penetrated faster from a buffer suspension at pH 7.0 into the acidic environment of *P. aeruginosa* biofilms than dendrons with OH or COO$^-$ groups at their periphery. In addition, dendrons with NH$_3^+$ peripheral groups accumulated near the top of the biofilm due to electrostatic double-layer attraction with negatively charged biofilm components. However, accumulation of dendrons with OH and COO$^-$ peripheral groups was more evenly distributed across the depth of the biofilms than NH$_3^+$ composed dendrons and exceeded accumulation of NH$_3^+$ composed dendrons after 10 min of exposure. Unlike dendrons with NH$_3^+$ groups at their periphery, dendrons with OH or COO$^-$ peripheral groups, lacking strong electrostatic double-layer attraction with biofilm components, were largely washed-out during exposure to PBS without dendrons. Thus, penetration and accumulation of dendrons into biofilms is controlled by their peripheral composition through electrostatic double-layer interactions, which is an important finding for the further development of new antimicrobial or antimicrobial-carrying dendritic polymers.

KEYWORDS: Nanomedicine, nanocarriers, nanotechnology, dendrimers, dendritic polymers

Biofilms are three-dimensional microbial aggregates responsible for 60−80% of all microbial infections. In an infectious biofilm, infecting organisms are protected by a matrix of self-produced extracellular polymeric substances (EPS), impeding effective penetration of most antimicrobials. This protection mechanism was already observed in 1684 by Antonie van Leeuwenhoek, describing how the vinegar which he used to wash his teeth only killed those bacteria which were on the outside of the scurf, nowadays called “biofilm”. To date, with the threat of antimicrobial-resistant bacterial infection becoming the number one cause of death by the year 2050, effective penetration of antimicrobials into biofilms is still a major hurdle in the treatment of infectious biofilms.

Dendritic polymers with dendrimers as the flagellar are flasiless and symmetrically branched macromolecules with a treelike structure. When composed of antimicrobial peptides, such dendrimers are able to kill planktonic bacteria, that is, suspended bacteria that are not in their protected, adhering, biofilm-mode of growth. Also antimicrobial dendrimers can prevent biofilm formation. For the treatment of existing infectious biofilms, dendrimers are under investigation for use as an antimicrobial nanocarrier. Vancomycin-tethered poly(amideamine) dendrimers showed avid binding to vancomycin-resistant *Staphylococcus aureus* surfaces. However, it is unclear whether the peripheral composition of dendritic nanocarriers stimulating avid binding to biofilm inhabitants is favorable or not for their deep penetration into an infectious biofilm. Dendrons are wedge-shaped structures that are the major component of dendrimers. These dendritic frameworks are inherently bifunctional containing one chemically addressable group designated to the focal point and a composition of multiple peripheral groups. Higher generation dendrons are by definition dendrimers with an active core and therewith the chemical composition of larger dendrons, similar to...
dendrimers, is responsible for efficient penetration in infectious biofilms. Considering the importance of penetration and accumulation of antimicrobials into an infectious biofilm and the promise of dendrimer-based antimicrobials for infection control, this work aims to determine the role of dendron peripheral composition in their penetration into Pseudomonas aeruginosa biofilms. P. aeruginosa causes a range of infections across the human body\textsuperscript{10} and is known to produce extensive amounts of EPS that can be especially troublesome in cystic fibrosis patients.\textsuperscript{7} Better understanding of the role of peripheral composition of dendrons in their penetration and accumulation into biofilm will aid their further development as an effective antimicrobial.

To this end, bifunctional dendrons were designed, consisting of rhodamine B as a red-fluorescent marker, an unsymmetrical triethylene glycol (TEG) linker, and three-generations (G3), multivalent 2,2-bis(hydroxymethyl)propionic acid (bis-MPA)
dendrons. Bis-MPA dendrons are biocompatible\textsuperscript{11} and biodegradable\textsuperscript{12} and can be synthesized with unprecedented structural control.\textsuperscript{13} Three different dendrons with pH-responsive peripheral composition were synthesized (see Figure 1a): rhodamine-TEG-G3-OH, rhodamine-TEG-G3-COO\textsuperscript{−}, and rhodamine-TEG-G3-NH\textsubscript{3}\textsuperscript{+} (charges indicated are as in the acidic environment inside a biofilm). Dendrons were constructed using conventional divergent growth of bis-MPA.\textsuperscript{14,15} Rhodamine B was covalently attached through fluoride-promoted esterification (FPE) chemistry and the peripheral hydroxyls were activated yielding the neutral dendritic scaffold, rhodamine-TEG-G3-OH. Esterification of rhodamine-TEG-G3-OH yielded anionic rhodamine-TEG-G3-COO\textsuperscript{−} and cationic rhodamine-TEG-G3-NH\textsubscript{3}\textsuperscript{+} (see Supporting Information for details).\textsuperscript{14,16} Complete substitution and high structural purity were corroborated using conventional characterization techniques for dendrimer chemistry, that is, NMR (Figure 1b) and MALDI-TOF-MS (Figure 1c).

Next, \textit{P. aeruginosa} ATCC 39324 biofilms were grown in a constant depth film fermenter (CDFF)\textsuperscript{17} to a thickness of 100 μm, as verified using optical coherence tomography (OCT result: 96 ± 15 μm, averaged across all biofilms employed in this study). Biofilms were exposed to red-fluorescently labeled dendrons suspensions for 0.1, 1, 10, or 100 min to study their penetration. In addition, biofilms after 100 min of exposure to a dendron suspension in phosphate buffered saline (PBS, pH 7.0) were subsequently placed in PBS without dendrons for another 100 min to monitor dendron wash-out. Note that in a separate experiment (data not shown), it was established that dendrons did not inadvertently release covalently coupled rhodamine in buffer, regardless of pH. After penetration and/or wash-out, biofilms were immediately embedded into Tissue-Tek OCT compound and flash-frozen in liquid nitrogen after which 10 μm sections were made perpendicular to the biofilm surface using a cryotome (Leica CM3050 S, Leica Microsystems, Wetzlar, Germany) for fluorescence microscopy.

Figure 2. (a) Cross-sectional images of \textit{P. aeruginosa} biofilms exposed to 0.2 μM dendron suspensions in PBS with different peripheral composition for 0.1, 1, 10, or 100 min and after 100 min wash-out in PBS (initial exposure time to dendron suspensions: 100 min). Identical alignment of the biofilms after embedding and cryo-sectioning was impossible, hence each fluorescence image is complemented with a light-microscopic image to visualize the entire biofilm (grasped within two arrows in the second image from the left, top row). The white scale bar represents 100 μm. (b) Enlarged overlay of fluorescence and light-micrographs of dendrons accumulated in a biofilm. Scale bar represents 100 μm. (c) A custom LabVIEW script was used to calculate the fluorescence intensity in a 10 × 0.645 μm (corresponding with one pixel) biofilm column as a function of biofilm depth (see also Experimental Section). (d) Example of fluorescence intensity as a function of biofilm depth, calculated as described in panel c, from which the depth-dependent dendron concentration was derived, using a standard curve (Figure S2).
Dendrons with NH$_3^+$ groups at their periphery accumulated faster into the acidic environment of P. aeruginosa biofilms than dendrons with OH or COO$^-$ at their periphery (Figure 3a,b), mostly accumulating near the top of the biofilms. Although initially penetrating less than dendrons with NH$_3^+$ groups at their periphery, after 10 min exposure the accumulation of dendrons with OH and COO$^-$ peripheral groups exceeded accumulation of dendrons with NH$_3^+$ peripheral groups in layers deeper than 30−40 μm into the biofilm (Figure 3c,d). Distribution of dendrons with OH and COO$^-$ peripheral groups was more even across the depth of the biofilms than of dendrons with NH$_3^+$ groups at their periphery. Importantly, neither the distribution (Figure 3e) nor the total concentration (Figure 3f) of dendrons with NH$_3^+$ groups at their periphery was affected by exposure to PBS, whereas dendrons with OH or COO$^-$ peripheral groups were largely washed-out during exposure to PBS.

Penetration and accumulation of antimicrobials, including antimicrobials transported by nanocarriers such as dendrimers, is a condition sine qua non for effective bacterial killing in infectious biofilms. Most studies on antimicrobial nanoparticle penetration and accumulation in biofilms have focused on the effects of charge, but outcomes are indecisive. Negatively charged nanoparticles are generally said to penetrate more easily into biofilms but oppositely also positively charged particles have been described to penetrate better into biofilms. Penetration depends on (1) the availability of transportation channels in biofilms with sufficient width to allow nanocarrier passage, (2) diffusion coefficients of the nanocarriers depending on their configuration and composition, and (3) their interaction with the channel walls, that is, the EPS matrix or bacterial cell surfaces. Dendrons are extremely small in the order of 2−5 nm, whereas water channel widths in biofilms are likely minimally 10-fold larger. Thus, size differences between the dendrons applied and thus their diffusion coefficients can be excluded as being causative to the difference in penetration and accumulation observed between the three different dendrons. Unfortunately, their small size did not allow reliable measurement of their zeta potentials; however, based on their pH-responsive peripheral composition it can be assumed that within an acidic P. aeruginosa biofilm (pH around 6.5) NH$_3^+$ groups ($\mathrm{pK_a}$...
around 9) will be protonated and positively charged, whereas COO\(^-\) groups (pK\(_a\) around 2) are deprotonated and negatively charged. The OH groups will remain uncharged inside \(\text{P. aeruginosa}\) biofilms. At the same time, EPS components\(^{26,27}\) and bacterial cell surfaces, including \textit{Pseudomonas} ones,\(^{28}\) remain negatively charged around pH 6.5. Thus, the accumulation of NH\(_3\)+ dendrons near the top of a biofilm can be explained by strong, electrostatic double-layer mediated adhesion of dendrons, impeding their penetration to deeper biofilm layers. The OH and negatively charged COO\(^-\) dendrons will migrate deeper into the biofilms, as they experience no electrostatic double-layer attraction with the channel walls and utmost weak Lifshitz-van der Waals attraction. Therewith the deeper penetration of neutral and negatively charged particles goes at the expense of being easily washed-out, an aspect frequently neglected in the current literature. We here show that prevention of dendron wash-out critically depends on their peripheral composition, that is, positive charged groups.

Carefully engineered chemistries of biomaterials or drug carriers, including antimicrobial nanocarriers, can become compromised when applied in the human body through interaction with components in the blood, mucus, and extracellular matrix of human tissue. Because the current study was designed to provide basic knowledge about the interaction of dendrons with biofilm rather than demonstrate in vivo performance, experiments were done in a buffer. In a protein rich environment, proteins will adsorb on dendrons depending on their peripheral composition,\(^{29}\) form a corona within 30 s, increase their hydrodynamic diameter, and make dendron zeta potentials less negative.\(^{29}\) Yet,\(^{12}\) surface-engineered nanoparticles possess the ability to permeate into cells,\(^{30}\) tumors,\(^{31}\) and pass the blood–brain barrier.\(^{32}\) Smart micellar nanoparticles with adaptive, pH-responsive engineered surfaces maintained their surface-engineered properties to penetrate and accumulate in biofilms in vivo after transport through the blood.\(^{2,30-38}\) This suggests maintenance of surface-engineered nanoparticle properties when applied under in vivo conditions. For more macroscopic biomaterials, it has been suggested\(^{30}\) in the past that proteins adsorbed and adjusted their conformation in a way that is maintained to reflect the chemistry of the underlying material; however, whether such an argument is valid to explain the abilities of engineered nanoparticles in vivo remains to be demonstrated.

In conclusion, penetration and accumulation of dendrons into biofilms is controlled by their pH-responsive peripheral composition. This conclusion offers better understanding of interactions between dendrimers and biofilm components during penetration and accumulation that is important for the development of new antimicrobial dendritic nanocarriers but in addition also offers the perspective of controlling the accumulation depth of dendrimers inside a biofilm. Although the dendrons in this study were labeled with rhodamine as a tool to measure their penetration, rhodamine can easily be replaced with more therapeutically potent antimicrobials. Most antibiotics have a similar size as rhodamine and can be coupled to the focal point of the dendron, alike the rhodamine, through a hydrolyzable ester or disulfide bond, provided the antibiotics have a similar hydrophobicity. Subsequently, the dendron can control the penetration and accumulation into a biofilm according to its peripheral composition. Together with their good biocompatibility,\(^{31}\) biodegradability,\(^{12}\) and commercial availability, that is, often the biggest obstacle in downward clinical translation,\(^{40}\) this warrants future exploitation of these dendrimers, as a new antimicrobial strategy for infection control.

### Experimental Section. Bacterial Strain, Growth Conditions, and Harvesting. \(\text{P. aeruginosa}\) ATCC 39324, an isolate from a cystic fibrosis patient, was grown aerobically for 24 h at 37 °C on a blood agar plate from a frozen stock and stored at 4 °C until use. One single colony was added to 10 mL of tryptone soya broth (TSB, Oxoid, Basingstoke, United Kingdom) and grown for 24 h at 37 °C after which the broth was added to 200 mL of TSB and incubated 16 h under rotary shaking at 150 rpm (37 °C). Bacteria were harvested by centrifugation for 5 min at 5000g, after which the bacterial pellet was washed two times with PBS (PBS, 10 mM potassium phosphate, 150 mM sodium chloride, pH 7.0). Bacteria were suspended in 200 mL TSB at a concentration of 5 \(\times\) 10\(^7\) bacteria per mL, as determined using a Bürker-Türk counting chamber.

#### Biofilm Growth in the Constant Depth Film Fermenter. Biofilms were grown on stainless steel disks in the constant depth film fermenter (CDIFF).\(^{17}\) Sterile stainless steel disks (diameter 5 mm) were placed in a pan, equipped with five wells with adjustable depth to house five disks. Fifteen pans were placed in the turntable of the CDIFF. The well-depth was set to allow growth of 100 μm thick biofilms with the aid of a scraper blade passing over each pan during rotation of the turntable at 3 rpm and maintaining the temperature inside the CDIFF at 37 °C. For inoculation of the disks in the CDIFF, 200 mL of bacterial suspension was dripped during 1 h on top of the pans, housing the disks. The turntable was stopped for revolving for 30 min, allowing the bacteria to adhere to the stainless steel disks, after which rotation was continued and artificial sputum medium\(^{17}\) was dripwise added at a flow rate of 16 mL h\(^{-1}\) on top of the pans and scraped across. After 18 h of biofilm growth, disks with adhering biofilms were aseptically taken out of the pans. One CDIFF run was comprised of 75 disks, from each run 15 randomly selected disk were taken from different pans for optical coherence tomography (OCT) analysis, and 30 biofilms were selected for dendron penetration experiments.

#### Optical Coherence Tomography. The thickness of biofilms was determined using OCT (Thorlabs Ganymade-II, Newton, NJ, U.S.A.). Biofilms were submerged in PBS, and three-dimensional scans of the complete biofilm were taken. OCT images were processed using a custom-made LabVIEW (National Instruments, Austin, TX, U.S.A.) script, which was corrected for background noise and possible tilting of the stainless steel disk surface. The average biofilm thickness was calculated using Otsu-thresholding of the image to determine the border between biofilm and surrounding fluid.\(^{12}\)

#### Dendron Penetration in Biofilms and Cryo-Sectioning of Biofilms. Dendrons were suspended to a concentration of 0.2 μM in PBS and 20 μL of a dendron suspension was pipetted over a biofilm surface. Dendron suspensions were spread evenly over the entire surface of the biofilm. Biofilms were exposed to dendron suspensions for 0.1, 1, 10, or 100 min, after which the biofilms were dip-washed in PBS. In addition, biofilms exposed for 100 min to a suspension of dendrons in PBS were transferred to PBS without dendrons for another 100 min to monitor dendron wash-out. Directly after washing, biofilms were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) and flash-frozen in liquid nitrogen. Next, biofilms...

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Fluorescent Imaging and Quantification of Dendron Penetration and Accumulation. Fluorescence microscopy (Leica DM 4000 B, Leica Microsystems Heidelberg Gmbh, Heidelberg, Germany) was carried out to image the biofilm sections. Fluorescence images were analyzed using a custom-built LabVIEW script to obtain the red-fluorescence intensity along the biofilm depth (see Figure 2c). The LabVIEW script first divided the biofilm image in vertical columns of 0.645 μm width (covering 1 pixel). Then, it aligned all the vertical columns with their tops along a straight line, after which the script calculated the average intensity profile as a function of biofilm depth. The dendron concentration in the biofilm was derived from the fluorescence intensity with a standard curve (Figure S2).

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**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available on free charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.9b00838.

Light- and fluorescence micrographs of rhodamine penetration into a 100 μm thick *P. aeruginosa* ATCC 39324 biofilm; synthesis of dendrons with different, pH-responsive peripheral compositions, calibration curves relating the fluorescent intensities to dendron concentration (PDF)

**AUTHOR INFORMATION**

**Corresponding Authors**

*(P.S.) E-mail: p.k.sharma@umcg.nl. Phone: +31503616097.*

*(M.M.) E-mail: malkoch@kth.se. Phone: +4687908768.*

**ORCID**

Henny C. van der Mei: 0000-0003-0760-8900
Michael Malkoch: 0000-0002-9200-8004
Prashant K. Sharma: 0000-0002-8342-8939

**Notes**

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