Antimicrobial Polymers of Linear and Bottlebrush Architecture: Probing the Membrane Interaction and Physicochemical Properties

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Abstract
Polymeric antimicrobial peptide mimics are a promising alternative for the future management of the daunting problems associated with antimicrobial resistance. However, the development of successful antimicrobial polymers (APs) requires careful control of factors such as amphiphilic balance, molecular weight, dispersity, sequence, and architecture. While most of the earlier developed APs focused on random linear copolymers, the development of APs with advanced architectures proved to be more potent in the mimicry of antimicrobial peptides. We recently developed multivalent bottlebrush APs with improved antibacterial and hemocompatibility profiles, outperforming their linear counterparts. Understanding the rationale behind the outstanding biological activity of these newly developed antimicrobials is vital to further improving their performance. This work investigates the physicochemical properties governing the differences in activity between linear and bottlebrush architectures using diverse spectroscopic and microscopic techniques. Linear copolymers are more solvated, thermo-responsive and possess facial amphiphilicity resulting in random aggregations when interacting with liposomes mimicking E. coli membranes. The bottlebrush copolymers adopt a more stable secondary conformation in aqueous solution in comparison to linear copolymers, conferring rapid and more specific binding mechanism to membranes. The advantageous physicochemical properties of the bottlebrush topology seem to be a determinant factor in the activity of these promising APs.

Introduction
Antimicrobial resistance (AMR) is recognized by the World Health Organization (WHO) as one of the top 10 global public health threats.[1] The daunting emergence of AMR is mainly a result of the misuse of antibiotics in humans and animals, and more alarming are the reportedly high rates of resistance to even last-resort antibiotics.[2,3] There is a very limited number of new antibiotic agents in the development pipeline,[3] and they are also most likely to face a rapid development of AMR once inducted on the market. An ineluctable post-antibiotic era is profiling in which the clinical treatment or prevention of an increasing number of infections is becoming almost impossible, particularly for immuno-compromised patients.[4-6] A favorable alternative that has been investigated to overcome AMR is the use of host defense peptides also known as antimicrobial peptides (AMPs).[7,8] They are a part of the innate immune system of virtually all multicellular organisms to selectively kill bacteria.[9] Owing to their mechanisms of action, ranging from membrane pore formation to complete membrane disruption,[8,11] AMPs have a low susceptibility to the development of AMR compared to traditional antibiotics.[12] Despite the broad spectrum and rapid onset activity of natural AMPs against various microorganisms, their labile nature, potential systemic toxicity, and high-cost production are impeding their use in clinical applications.[7]
Antimicrobial polymers (APs) mimicking the prominent features of AMPs have been designed in the last decades to circumvent the limitations of AMPs. To achieve optimal antimicrobial activity, which is based on the disruption of bacterial cells, a number of important structure-activity relationship principles need to be carefully considered in the design of AMPs mimics. The most universal principle is the balance between cationic and hydrophobic (and hydrophilic) components of the polymer. In the cationic unit is essential to initiate the selective binding with negatively charged bacterial cell membranes through electrostatic interactions, and subsequently, the hydrophobic moieties can insert into the lipid bilayer and cause its disruption. In addition to the amphiphilic balance, other interconnected properties that may affect the antimicrobial activity, as well as the toxicity profile of the APs, must be carefully considered in the structure design. Among others, the molecular weight, the dispersity, the nature of the cationic unit, the spacial organization of subunits, the properties of the end group, the monomer sequence, and the chain architecture play a role.

The versatility of polymers and advances in polymerization techniques have allowed the synthesis of AMP mimics of various architectures playing a determinant role in their biological activity. While earlier studies demonstrated the advantages of random, linear (co)polymers, recently developed design of polymers such as (multi)blocks with a controlled sequence of monomers, hyperbranched structures, dendrimer-based nanostructures, or spherical and rod-like polymer bottlebrushes have proven to possess improved antibacterial activity, minimal hemolytic toxicity, and target specificity. Recently, we could demonstrate that multivalent presentation of linear antimicrobial polymers as grafts in a bottlebrush copolymer could drastically improve the selectivity of the system as it boosted antibacterial activity while simultaneously reducing unspecific interactions with red blood cells. In this regard, it is worth looking beyond the conventional structural factors in the design of APs and exploring the physicochemical properties of linear and bottlebrush architectures to derive mechanistic details of their interaction with bacterial membranes. This will lead to unraveling/formulating the structure-activity relationship principles governing the increased performance of bottlebrush polymers.

Hence, this work is aimed at elucidating the intrinsic physicochemical characteristics of two copolymers (linear and bottlebrush copolymers with identical composition) and their mechanisms of interaction with bacterial membrane models. The intrinsic physicochemical properties governing the difference in activities between the bottlebrush copolymer (BS40) and the linear copolymer (S40), were analyzed by aqueous size exclusion chromatography (SEC), Fourier transform infrared spectroscopy (FTIR), and potentiometric measurements. Large unilamellar vesicles (LUVs) as well as supported lipid bilayers mimicking Escherichia coli (E. coli) gram-negative bacteria and red blood cells (RBC) were used as membrane models to investigate the interaction with the two APs by means of fluorescence spectroscopy, dynamic light scattering (DLS), quartz crystal microbalance with dissipation (QCM-D) and cryo-electron microscopy (Cryo-EM).
Table 1. Analytical data of linear and bottlebrush copolymers.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Aqueous SEC of deprotected polymers</th>
<th>pH Titration</th>
<th>Dye leakage fluorimetry assays</th>
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<tr>
<td></td>
<td>PVP Calibration</td>
<td>LS calibration</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; [µg mL&lt;sup&gt;-1&lt;/sup&gt;]</td>
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<tr>
<td></td>
<td>M&lt;sub&gt;n&lt;/sub&gt; [g mol&lt;sup&gt;-1&lt;/sup&gt;]</td>
<td>D</td>
<td>M&lt;sub&gt;n&lt;/sub&gt; [g mol&lt;sup&gt;-1&lt;/sup&gt;]</td>
</tr>
<tr>
<td>S40</td>
<td>3,600 1.35 - - - 8.0 279 &gt; 2,500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BS40</td>
<td>33,000 1.32 100,000 1.16 0.135 7.5 344 &gt; 2,500</td>
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(M<sub>n</sub>: molecular weight, D: dispersity, dn/dc: refractive index increment, pK<sub>a</sub>: acid dissociation constant, EC<sub>50</sub>: effective concentration producing 50% of the maximal response)

Results and Discussion

We recently developed multivalent bottlebrush copolymers using two orthogonal polymerization techniques: viz. reversible addition-fragmentation chain-transfer (RAFT) polymerization and ring-opening metathesis polymerization (ROMP). Briefly, RAFT polymerization, using a norbornene-functionalized chain transfer agent (CTA), was first used to synthesize macromonomers with a statistical distribution of N-isopropylacrylamide (NiPAAm) and aminoethylacrylamide (AEAm) in its protected form, i.e. N-tert-butoxycarbonyl-N'-acryloyl-1,2-diaminoethane (BocAEAm). Then, ROMP was applied to produce bottlebrush copolymers using a Grubbs catalyst. Boc-deprotection was then performed to free the primary amino functions of the polymers, necessary to interact with negatively charged bacterial membranes (Scheme 1). In general, the multivalent presentation in bottlebrush-like architecture was found to improve the antibacterial activity and reduce the hemotoxic potential of the statistical macromonomer. One of the synthesized bottlebrush copolymers, BS40 (comprising 40% of cationic comonomer), with a statistical distribution of monomers in the grafts was particularly interesting. BS40 showed outstanding broadband antimicrobial activity against gram-positive as well as gram-negative bacteria and improved hemocompatibility compared to its linear counterpart, the statistical copolymer S40. The structure of synthesized polymers can be found in Scheme 1. Noteworthy, BS40 and S40 were identical considering the overall ratio of NiPAAm and AEAm and the difference in their activity could only be associated with the architecture and thus they were chosen for further physicochemical studies.

Size exclusion chromatography (SEC)

In our previous study, SEC was performed in tetrahydrofuran (THF) using poly(styrene) as calibration on polymers before deprotection. While the analysis revealed narrow distribution for the synthesized polymers, the prediction of the molecular weight of the bottlebrush was handicapped by the presence of unreacted macromonomers and in addition, comparison with a linear calibration could not be accurate for bottlebrush copolymers. In addition, after Boc deprotection, bottlebrush copolymers were filtered from the residual small molecules and any ungrafted linear chains by ultra-centrifugation. Thus we set out to confirm the successful deprotection and purification of the polymers via aqueous SEC (Figure 1). A molecular weight (M<sub>n</sub>) of 3,600 g mol<sup>-1</sup> was obtained for the linear S40 using a poly(vinyl pyridine) (PVP) calibration, whereas an M<sub>n</sub> of 33,000 g mol<sup>-1</sup> was obtained for BS40 using the same PVP calibration (Table 1). The latter value was, however, still inaccurate as the bottlebrush nature of the polymer is not reflected by the linear calibration standard.

We, therefore, used a multi-angle laser light scattering (MALLS) calibration, which is more appropriate for estimation of molecular weight distribution for polymers with non-linear architectures, and the deprotected bottlebrush BS40 was found to have an M<sub>n</sub> of 100,000 g mol<sup>-1</sup>. Considering the M<sub>n</sub> of the macromonomer S40 and bottlebrush BS40 obtained with PVP and MALLS calibrations, respectively, an average DP of 28 could be inferred for the BS40, which was close to the targeted DP of 25.

Figure 1. SEC curves of linear macromonomer S40 (in blue) and bottlebrush copolymer BS40 (in green) in aqueous eluent. Solid lines represent the molar mass distribution of polymers using poly(vinyl pyridine) (PVP) as calibration and the dotted line shows the distribution with multi-angle laser light scattering (MALLS) calibration.
Contrary to previous findings reporting that higher molecular weight may reduce the suitability of polymers in an antimicrobial context,\cite{20} the increase in molecular weight through ROMP of the macromonomer was rather beneficial as the resulting bottlebrush copolymers showed better selective antibacterial behavior compared to the linear copolymers.\cite{32}

However, the hydrodynamic behavior of bottlebrush copolymers is vastly different when compared to the behavior of linear chains of similar length. Considering that only the mass concentrations were used for bioassays, this difference in bioactivity could only be explained by the specific architectural conformation of the brush polymers. This triggered our attention to further study the physicochemical differences between the linear and brush polymers, particularly when solvated.

**Structural analysis by Fourier transform infrared spectroscopy (FTIR)**

As antimicrobial biopolymers are expected to exert their activity under aqueous physiological conditions, it is important to investigate the differences in hydration and solvation between the linear and bottlebrush architectures in an aqueous solution.

FTIR spectroscopy is a well-established analytical tool frequently used to dissect the structural information of chemical and biological molecules on the molecular level.

Here, important to invoke is the amide band in FT-IR spectra of proteins, composed of mainly C=O stretching vibration and smaller contributions of C-N stretching and N-H bending of the amide bond, that is often referred to as the most sensitive marker band to secondary structure elements due to its sensitivity to H-bonding environment in polypeptides and proteins.\cite{33,34} Both the antimicrobial biopolymers S40 and BS40 contain prominent amide bonds in the side chain of the monomers forming the polymer structure (see Scheme 1).

FTIR spectra were recorded for the linear and bottlebrush copolymers, S40 and B40 respectively, which were dissolved in D$_2$O phosphate buffer and DMSO. In the spectral region of interest ranging from 1700-1400 cm$^{-1}$, signals between 1700-1600 cm$^{-1}$, originating primarily from C=O stretch vibrations referred to as amide I band in proteins, and at 1580-1350 cm$^{-1}$ from combinations of NH bending and C-N
stretching modes (referred to as amide II band in proteins) were detected for all polymers studied (Figure 2).[35]

We first analyze spectra of polymers dissolved in D$_2$O. Data were compared to FTIR spectra of the homopolymers NIPAAm (PNiPAAm) and AEAm (PAEAm) to inspect how polymerization affected the individual structures of monomers. (see SI Figure S1 and Scheme S1) and to support assignment of spectra signatures. Indeed, spectra of S40 and BS40 could be composed of individual spectra of monomers, albeit small differences are present in the region 1600-1650 cm$^{-1}$ which will be further discussed.

In the region from 1600 - 1700 cm$^{-1}$, one narrow peak of higher intensity and broader peak of smaller intensity is detected in FTIR spectra of both polymers, albeit of different relative intensity. When APs are dissolved in D$_2$O, the former occurs at 1672/1674 cm$^{-1}$ and the broader peak is most likely a superposition of several contributing vibrational modes the range of 1617-1652 cm$^{-1}$, which are relatively more intense in the BS40 spectrum (Figure 2A). The contribution of an ensemble of different modes leading to multiple peaks in this region is most likely a reflection of the heterogeneity in H-bonding interactions with C=O oscillators, attributed to slightly different structural elements. Although the assignment of individual species to structural components is beyond the scope of the manuscript, structural differences between the two polymers can be derived from the relative differences of spectra and by comparison to the spectra of the individual monomers (SI Figure S1). We assign the more intense peak at 1672 cm$^{-1}$ to the C=O stretch vibration of the C=O groups in the AEAm units, whereas the broader peak at 1617-1652 cm$^{-1}$ (with max. intensity at 1635 cm$^{-1}$) originates mostly from the C=O oscillators in the NIPAAm units with some contributions from AEAm as well (compare to SI figure S1).

Stronger H-bonding to any C=O oscillator results in larger anharmonicity thereby leading to a lower observed vibrational frequency, hence we conclude that increased intensity of the broad peak at 1635 cm$^{-1}$ in BS40 in comparison to S40 is reflecting more structured H-bonding to the C=O groups in BS40, most likely in the AEAm units. We suggest that the primary amine from ammonium (in the AEAm unit) can act as an H-bonding partner to C=O groups in the BS40, mainly within the AEAm units. Such interaction might result in an overall more defined and stabilized structure of the BS40 bottlebrush architecture as intra-brush H-bonds occur, which in contrast are limited sterically in S40.

FTIR spectra of BS40 and S40 dissolved in the aprotic solvent DMSO (Figure 2b) reveal the differences in solvent accessibility and H-bonding to the polymers. All peaks assigned to C=O oscillators are shifted to higher wavenumbers (see also Fig. S2) reflecting the loss of hydrogen bonding to the solvent. Furthermore, the intensity difference for the peak assigned to the C=O oscillators in NIPAAm (1635 cm$^{-1}$ in D$_2$O, 1653 cm$^{-1}$ in DMSO) between BS40 and S40 is much lower for DMSO, further supporting our interpretation that BS40 is stabilized by intra-brush H-bonds, which are destabilized in DMSO. Of particular interest is the negative difference for peaks in the region 1400 - 1500 cm$^{-1}$, showing a higher intensity of DMSO modes in the S40 spectrum, i.e., S40 is more solvated by DMSO than BS40. A comparison of the spectra of S40 in DMSO and D$_2$O further confirms that S40 is more solvated by DMSO than BS40 (Figure S2). This could be explained by the fact that the S=O functional groups of the solvent act as H-bond acceptors from the ammonia group in S40, whereas in BS40 those groups are involved in intra-brush H-bonds and therefore do not contribute to solvent bonding.[36]

None of the herein investigated polymers showed a cloud point behavior in a relevant temperature region in water.[32] This is expected as the hydrophilic nature of the cationic charges is likely preventing phase separation. Still, a thermo-responsive behavior leading to a change in solvation could be expected from the polymers, as PNiPAAm has a documented thermo-responsiveness.[37] Hence, we have performed and evaluated the structural differences of polymers as a response to elevated temperature by FTIR spectroscopy.

Experiments were performed in D$_2$O buffer in a temperature range from 25 °C to 75 °C (with an increment of 5 °C per step). The reversibility of potential transitions was probed by measuring after cooling down to 25 °C. The spectra revealed a remarkable difference in the thermal behavior of the two APs architectures (Figure 3A and 3B). The concomitantly monitored peaks at 3420 cm$^{-1}$ and 1674 cm$^{-1}$ corresponding to water (Figure S3) and C=O stretch vibrations, respectively, clearly showed that on one hand, the linear APs was characterized by a phase transition marked by a complete and irreversible disappearance of the vibrational modes, with an inflection point observed at about 57 °C (Figure 3C). This could be explained by dehydration of the hydrophobic repeating unit, NIPAAm, which has a well-known lower critical solution temperature (LCST). The onset of the thermal response is higher when compared to pure PNiPAAm, which is likely associated with the presence of the hydrophilic comonomer. However, the AEAm cationic units could stay hydrated to keep the whole linear polymer S40 solubilized, suggesting that the S40 is more likely governed by a facial amphiphilicity in aqueous media.
On the other hand, a mostly reversible decrease of about 18% in the spectra intensity of the vibrational modes of the bottlebrush BS40 could be observed (Figure 3D). This could be explained by the densely packed architecture and H-bond stabilized structure of the brush structure which might be pivotal to keep both NiPAAm and AEAm hydrated. Hydration of the NiPAAm in BS40 could be confirmed by the broad and less intense peak appearing around 1559 cm$^{-1}$ which was not noticeable in the spectra of S40 (Figure S1).

Overall, the FTIR results suggested a more stabilizing secondary conformation of the bottlebrush copolymer BS40 with a less pronounced and reversible thermal transition in an aqueous solution. As hydrogen bonding is thought to be involved in membrane attachment of antimicrobial polymers, this difference could be an important contributor to the improved biological activity of bottlebrush copolymers.

**Potentiometric titration**

The acid dissociation constant (pK$_a$) is an important physicochemical parameter that can influence the biological activity of compounds as ionization states can affect their pharmacokinetic profiles including the interaction with membranes. The results of potentiometric titration of the two polymers indicated a slight difference in their pK$_a$ (Table 1 and Figure S4). The linear S40 was expected to undergo more deprotonation of the ammonium group by titration with NaOH, consequently resulting in a higher apparent pK$_a$ of 8.0. For BS40, the densely packed architecture in brush architecture could be responsible for the slight decrease in the apparent pK$_a$ up to 7.5.
Figure 3. Concentration-dependent dye leakage from E. coli-liposomes interacting with S40 (A) and BS40 (B) and from RBC-liposomes incubated with S40 (C) and BS40 (D). Data are presented as normalized percentages. The initial baseline (0% of dye leakage) corresponds to the fluorescence of liposomes before the addition of polymers and the 100% reflects the maximum fluorescence after disruption of liposomes by the addition of the detergent Triton X. All curves were smoothed at 40 points of window by the Savitzky-Golay method.

This could be explained by potential electrostatic repulsion between neighboring macromonomers’ charges, and also by the presence of H-bonding between C=O and ammonium groups, as demonstrated by FTIR spectroscopy results, which could both disfavor protonation of the ammonium group. The linear copolymer is slightly more basic than the bottlebrush. However, both S40 and BS40 (with pK<sub>a</sub> > 7.4) should stay protonated in the physiological conditions, which is relevant for their respective antimicrobial bioactivities. Still, when considering the virtually identical chemical composition of the polymers, the difference in pK<sub>a</sub> values illustrates the influence of the polymeric architecture on the functional properties of the macromolecules, which may be relevant in the polymer-membrane interactions at physiological conditions.

**Dye leakage assays using Liposomes**

Liposomes, which are spherical vesicles made of a bilayer of phospholipids have been widely used as simplified model membrane systems to study the interaction between antimicrobial polymers and phospholipid membranes. Similar to AMPs, the electrostatic polymer-membrane interaction is vital for initiating antimicrobial activity. Phospholipids are elementary components that define the net charge of most bio-membranes and are, depending on the type of phospholipids, important factors of distinction between membranes of prokaryotic and eukaryotic cells. In the herein conducted study, two types of liposomes were prepared, viz., E. coli mimicking liposomes as gram-negative bacteria model and RBC mimic liposomes as eukaryotic cells model.

LUVs mimicking the E. coli membranes were prepared by mixing 80% of zwitterionic lipid, 2-Oleoyl-1-palmitoyl-sn-glycero-3-phosphoethanolamine (POPE), and 20% anionic lipid, 2-Oleoyl-1-palmitoyl-sn-glycero-3-phospho-L-serine (DOPS) which is an anionic lipid, were used as a proxy for red blood cell membranes. Calcein, here
used as a fluorescent dye, was encapsulated in the liposomes at a self-quenching concentration. The addition of polymer solutions of different concentrations to the LUVs suspension caused membrane permeabilization which was traced by fluorescence intensity. The release of calcein upon membrane disruption dilutes the concentration of the dye in the liposomes, leading to increased fluorescence intensity in the media. Time-resolved dye-leakage measurements were undertaken to investigate the permeability of liposomal membrane caused by antimicrobial polymers.

For liposomes mimicking the membrane of *E. coli*, both S40 (Figure 4A) and BS40 (Figure 4B) induced dye leakage in a concentration-dependent manner of up to 100% of dye released at high concentrations within 10 min. Surprisingly, the S40 and BS40 had very similar dye leakage EC$_{50}$ of 279 µg mL$^{-1}$ and 344 µg mL$^{-1}$, respectively, with the difference within the error limits of the method (Table 1 and Figure S5). It is important to mention that in our previous study the BS40 showed improved antibacterial activity against *E. coli* bacteria with a minimum inhibitory concentration (MIC) of 64 µg mL$^{-1}$ compared to S40 (MIC = 128 µg mL$^{-1}$). However, the EC$_{50}$ of polymers inducing dye leakage from liposomes cannot be directly translated into the MIC. In fact, the EC$_{50}$ in this context is a value assessing the membrane permeability of artificial membranes, whereas the MIC in bioassays evaluates the growth inhibition of the actual living bacteria, comprising far more complex membrane compositions.

A concentration-dependent dye release was also observed for RBC-liposomes (Figure 4C and 4D), however, 50% of dye leakage could not be reached for both S40 and BS40 even at the higher concentration tested (Dye leakage EC$_{50}$ > 2500 µg mL$^{-1}$). Nevertheless, the overall dye leakage activity of BS40 seems to be increased compared to S40. It should be noted that in the initial study of hemocompatibility, hemolysis did not play a significant role and the parameter used to determine blood compatibility was hemagglutination (polymer induce blood clotting).

Overall, the dye leakage experiments using liposomal membranes correlate with the previously reported hemocompatibility properties, with both the macromonomer S40 and the corresponding homograft bottlebrush BS40 showing a higher affinity to bacterial cell-like membranes. This suggested that the dye leakage was neither dependent on the architecture nor the molecular weight but was rather dependent on the type of membrane used. The superior selectivity of the APs towards *E. coli*-lipoosomes compared to RBC-lipoosomes could be explained by the negative intrinsic curvature of phosphatidylethanolamine lipid, predominantly present in *E. coli* bacteria, which promote membrane permeation upon interaction with antimicrobials.

*Quartz Crystal Microbalance with Dissipation (QCM-D)*

The polymer-membrane interaction is generally governed by electrostatic interaction which allows binding of antimicrobial polymers to membrane surfaces. However, the molecular adsorption, as well as the binding kinetics of the polymers to the surface, could not be quantified in real-time on LUVs with the above-used techniques. We, therefore, used QCM-D to further investigate the interface dynamic of the APs with supported planar bilayers made of phospholipids as the main components of cell membranes.

![Figure 5](https://example.com/qcm_d.png)

**Figure 5.** Typical QCM-D measurements data (A) for model membranes mimicking RBC-liposomes, where dash lines showing the frequency $\Delta f$ and solid lines the dissipation $\Delta D$ varying at different steps namely, [I] the injection of liposomes to form supported lipid bilayer (SLB), [II] the rinsing of extra lipids with PBS, [III] the injection of polymers S40 and BS40 (156 µg mL$^{-1}$), [IV] the rinsing off first with PBS, [V] and then with milliQ water. The mass per area (m/a) showing the adsorption (B) and desorption (C) profiles of polymers on the supported bilayer over time, the time of injection of polymer is considered as initial time (Time= 0 second). The data were fitted with Equation 3 (for details see experimental parts).
The RBC-liposomes, composed of 90% zwitterionic phospholipid, DOPC, and 10% anionic phospholipid, DOPS, formed a supported lipid bilayer (SLB) (Figure 5A) by spontaneous decomposition of vesicles on the silicon oxide sensors crystals used as substrate. However, under our experimental conditions, the vesicles made of POPE (80%) and POPE (20%) as E. coli bacterial membrane mimics could not form complete SLB (Figure S7). For this reason we have only analyzed the kinetic traces of adsorption of the polymers on the former. The presence of 10% of negatively charged phospholipid in RBC-liposomes is sufficient to induce electrostatic interaction and consequently binding of polymers on the SLB.

Upon the addition of the polymers to the SLB, a rapid binding to the surface could be observed for both polymers. The mass of polymers adsorbed (Figure 5B), as well as the chain density (Figure S8) per surface area, were deduced based on the modified Sauerbrey relation. The mass of bottlebrush copolymer BS40 adsorbed was about three times higher and twice faster compared to the linear S40. The multivalence of the bottlebrush architecture seems to be advantageous for surface binding. This is also in accordance with previous investigations on the membrane interaction of these polymers with bacteria, where BS40 also showed a drastically improved kinetic. Further, data yield that both polymers do not show a dynamic exchange (adsorption vs desorption) in PBS, due to rinsing with PBS (Figure 5A, IV) the polymers remain adsorbed on the SLB. This indicates that already adsorbed polymers are strongly bound at the SLB, but they desorb when flushing the chamber with MilliQ water (Figure 5A, V) indicating a dynamic exchange behavior in MilliQ water. For the latter, the desorption rate is twice slower for BS40 in comparison to S40 and supports the higher binding tendency of BS40 (Figure 5C).

When considering the molecular weights of each polymer obtained by SEC (viz., 3,600 g mol⁻¹ and 100,000 g mol⁻¹ for S40 and BS40, respectively), the results in terms of chain density clearly showed that the number of linear-S40 chains adsorbed at the surface is approximately 10 times higher than the number of brush-BS40 chains (Figure S8). This would suggest that the linear polymers get attached to liposomes' surface in a higher numbers and in an unspecific way that could promote aggregation, unlike the bottlebrush structures that are less flexible and less solvated. Hence, the bottlebrushes which are adsorbed with high chain density/surface have a higher impact on the membrane integrity.

![Figure 6](image-url)  
Figure 6. DLS radius distribution of E. coli-liposomes alone (A), and liposomes incubated with 20% Triton X (B) and with S40 (C) or BS40 (D) at the concentration of 156 µg mL⁻¹. A spectroLight 610 was utilized to study the liposomes aggregation and data are presented as normalized amplitudes of the recorded radius.
Dynamic Light Scattering (DLS)
As already mentioned, membrane destruction is not the only relevant mode of interaction, and in particular, in the context of blood compatibility, agglutination plays an important role as well. To investigate this aspect further, *E. coli* liposomes were incubated with the two polymers (S40 and BS40) and with Triton X. While the detergent, Triton X-100 (20%), used as control induced disruption of LUVs, both polymers at the concentration of 156 µg mL⁻¹ induced aggregation of the vesicles instead of destruction of LUVs. Intriguingly, the linear copolymer, S40, generally induced considerably larger aggregates compared to the bottlebrush polymer, BS40 (Figure 6).

The linear copolymer S40 has adopted irregular conformations when interacting with the membrane surfaces due to its increased facial amphiphilicity; while the hydrophobic units could disturb the membrane and induce dye leakage, the cationic units at the interface with the aqueous environment (highly solvated) could interact electrostatically with neighboring liposomes and therefore result in random aggregation. In contrast, the bottlebrush structure with more stable conformation, i.e., a more pronounced secondary structure and less solvated properties, as demonstrated by FTIR results, have exerted dye leakage but seemingly with a much more specific membrane interaction. This might be advantageous for selectivity and correlates with our previous results where the BS40 proved to be less hemagglutinating.

Moreover, kinetics DLS measurements performed in the same time frame as the dye leakage experiments and at different concentrations revealed an obvious difference in the hydrodynamic distribution of *E. coli*-LUVs based on their interaction with the polymers (Figure S6).

While the dye leakage experiments did not show significant differences between the linear and bottlebrush copolymers, DLS results clearly show that they do interact differently with membranes models. This is further supported by the distinct physicochemical characteristics of the two polymers already discussed and suggests different mechanisms of membrane interaction.

Cryogenic Electron Microscopy (Cryo-EM)
Transmission electron cryo-microscopy is a powerful microscopy technique to study the liposomes in their native state as it can provide detailed information on the lamellarity, size, and shape of the vesicles. In this study, the impact of the linear S40 and bottlebrush BS40 polymer on the shape of the *E. coli*-LUV was investigated using cryo-EM.

Before freezing and cryo-electron microscopy screening, the liposomes were incubated with the polymers (S40 and BS40) for 10 min, at the polymer-concentration of 156 µg mL⁻¹ that proved to be sufficient for membrane adsorption in QCM-D experiments. The cryo-EM images obtained (Figure S9) showed generally a distinction in the shape distribution of treated and untreated liposomes. More interestingly, statistical analysis of the vesicles from the cryo-EM images revealed that the BS40 induced significant changes in the aspect ratio of the liposomes’ population (*n*=800), as shown in Figure 7, compared to their linear counterparts. This could be explained by the multivalence and high density of the bottlebrushes. In fact, they are adsorbed at the membranes at higher mass per surface area, as demonstrated by QCM results, and consequently exert a higher impact on the liposomes resulting in a change of shape of vesicles. In addition, the elongated and stiff nature of bottlebrush copolymers is more likely to have an impact on the membrane morphology in a long-range (size of the LUV). This suggests a unique and more efficient mechanism of action of the bottlebrush architecture correlating to their improved antimicrobial activity on *E. coli* bacteria as previously reported.

![Figure 4. Cryo-EM images of *E. coli*-liposomes alone and *E. coli*-liposomes incubated with linear S40 and bottlebrush BS40. The changes in the shape of liposomes on the images were statistically examined and the aspect ratio (height divided by width) tendency of the liposomes (*n*=800) was inferred for each case.](image)

Conclusion
The physicochemical behavior of linear and bottlebrush polymeric antimicrobial peptide mimics, composed of the same overall ratio of cationic and hydrophobic monomers,
and their interaction with liposomes membranes were investigated. The comprehensive range of analytical techniques used in this study complementarily proved the differences in the structure-activity relationship between the two polymer architectures.

FTIR revealed that the densely packed bottlebrushes had a more stabilized secondary structure due to the presence of H-bonding and were less hydrated when compared to their linear analogs. Dye leakage experiments from liposome models using fluorescence spectroscopy confirmed the selective activity of both polymers towards E. coli-liposomes membranes, while polymer architecture did not result in drastic differences in this context. A surprising result, given the differentiated activity which was reported in biological tests. QCM-D revealed that the binding of the densely packed bottlebrush structures was more efficient on lipid bilayers. Moreover, dynamic light scattering indicated two distinct hydrodynamic distributions with random aggregation obtained for linear APs whilst the bottlebrushes resulted in moderate aggregation as opposed to the complete disruption induced by a surfactant. Finally, cryo-EM measurements revealed a shape change of LUVs in the presence of bottlebrush copolymers, likely a result of their elongated and confined nature.

Unlike linear copolymers that showed a certain facial amphiphilicity behavior, the bottlebrushes with their stable conformation might possess a global amphiphilicity with less propensity to “bury” the hydrophobic domains. In addition to the high density of cationic charges, the antibacterial potential of bottlebrush topology may be improved. In accordance with our previous studies that reported an improved antibacterial activity and hemocompatibility for bottlebrush copolymers, the bottlebrushes were found to possess advantageous physicochemical properties conferring a more defined mechanism of action to the polymers.

Overall, the confined nature of the bottlebrush architecture seems to have beneficial effects on the molecular level (hydration, H-bonding), as well as in a larger size range (shape change and aggregation of LUVs). As a result, future synthetic work should investigate the influence of this confinement in a more differentiated way, e.g., by systematically varying the grafting density in antimicrobial bottlebrush copolymers. Moreover, as the side chain length might impact phase behavior and solubility, reducing the length of linear building blocks could be considered to limit the degrees of freedom of bottlebrush architectures even further and therefore to potentialize their activity.

**Experimental Section/Methods**

**Size exclusion chromatography (SEC)**

Aqueous Size-Exclusion Chromatography was measured on an instrument from PSS (Mainz, Germany) equipped with RI and UV (280 and 309 nm), as well as a 7-angle MALLS detector (SLD 7000). The stationary phase consisted of a set of NOVEMA Max columns (1 x 30 Å, 8 x 3000 mm; 2 x 1000 Å, 8 x 300 mm). HPLC grade water with TFA (0.3 vol %) and NaCl (0.1 mol L⁻¹) was used as the eluent after filtration and poly(vinyl pyridine) as calibration. For MALLS measurements, dn/dc was determined by performing different injections varying the injections volume and thus the injected sample amount. The area under the RI curve was integrated and fitted against injected mass to determine the dn/dc (slope of the linear regression).

**Fourier-transform Infrared spectroscopy (FTIR)**

The samples were dissolved in D₂O with phosphate buffer (20 mM), if not indicated differently, to reach a final concentration of 50 mg mL⁻¹. FTIR measurements were performed in a transmission mode on Bruker Tensor II equipped with a liquid-nitrogen-cooled photoconductive MCT detector. The spectra were recorded in the range of 800 – 4000 cm⁻¹ with a spectral resolution of 2 cm⁻¹. 256 scans were recorded and averaged for every IR spectrum. The temperature-dependent measurements were prepared using a water heater in the following jump: 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, and 25 °C. Spectra were recorded after 5 min of equilibration time after setting a certain temperature. The spectra were baseline corrected, normalized to amide I peak and analyzed using OPUS software.

**Potentiometric titration**

The potentiometric titration cell was a 10 mL scintillation vial with a general-purpose pH probe connected to a Mettler Toledo pH meter. Before each experiment, the pH meter was thoroughly washed with deionized water and calibrated with standard buffers (pH 4.0, pH 7.0 and pH 10.0). All solutions were prepared with deionized water and titrations were carried out at ambient temperature (25 °C) and under gentle mixing with a magnetic stirrer.

The titrate solution (3 mL) prepared at a concentration of 1 mg mL⁻¹ of polymers in HCl (0.001 M) was placed in the titration cell. The titrant solution, freshly prepared NaOH (0.1 M), was gradually added with Eppendorf micropipettes (from 20 µL to 540 µL). Sufficient time (about 1 min) was necessary to reach a stable pH reading before the next addition of the base. The recorded pH values were plotted against the cumulative volumes of the titrant. The titration
curve and its first derivative were used to determine the equivalence points as well as the pKₐ.

**Preparation of liposomes**

A first buffer (buffer A) was prepared by dissolving Na₂HPO₄ (142 g, 1 mmol) in milliQ water (90 mL). The pH was adjusted to 7.0 and the volume was then topped up to 100 mL with milliQ water. To obtain the calcein solution (40 µL), calcein (249 mg, 0.3 mmol) was suspended in previously prepared buffer A (8 mL). A solution of NaOH (1 M) was added dropwise to dissolve the calcein and the pH was adjusted to 7.0. The total volume was then topped up to 10 mL with buffer A.

A second buffer (buffer B) was prepared by dissolving Na₂HPO₄ (142 g, 10 mmol) and NaCl (5.26 g, 90 mmol) in milliQ water (980 mL). The pH was adjusted to 7.0 and the solution was then taken up to 1000 mL with milliQ water.

Different mixtures of phospholipids were used to prepare liposomes mimicking membranes of E. coli and red blood cells. For E. coli-liposomes, 2-Oleoyl-1-palmitoyl-sn-glycero-3-phosphoethanolamine (6 mg, 8.4 mmol) and 2-Oleoyl-1-palmitoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (1.6 mg, 2.1 mmol) were dissolved in of CHCl₃ (0.8 mL) in a 25 mL round bottom flask. And for liposomes mimicking red blood cells a mixture of 1,2-dioleoyl-sn-glycero-3-phosphocholine (9 mg, 123 mmol) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (1 mg, 1.23 mmol) were dissolved in CHCl₃ (1 mL). A thin film lipid was formed by drying the organic solvent on the Rotavap under vacuum. The dried thin film was then hydrated with calcein solution (1 mL) or phosphate buffer saline (1 mL) for 1 hour under continuous stirring. The round bottom flask was then subjected to 5 freeze-thaw cycles in liquid nitrogen for 5 min and then in a water bath at 25 °C for 15 min to improve the encapsulation of calcein. The hydrated lipids were extruded 15 times using polycarbonate membranes of 400 nm. Particularly for QCM-D and TEM, empty liposomes made with PBS as hydration media were additionally extrud 15 times through 100 nm membranes.

To purify the calcein-loaded liposomes from the non-encapsulated dye, the liposomes suspensions were diluted in buffer B and then concentrated by centrifugation at 4000 xg using an Amicon Ultra-15, PLTK Ultracel-PL Membran, 30 kDa for 50 min. The purification process was repeated at least 3 times. The concentrated liposomes suspension was stored at 4 °C and used within one week.

**Dye leakage experiment**

Kinetics acquisition (time-based) measurements of polymer-induced dye leakage from the liposomes were performed on a Fluoromax 4 spectrofluorometer (Horiba, USA). The excitation wavelength was set at 490 nm (slit: 1.0 nm Bandpass) and the fluorescence intensity was monitored over time 800 seconds at the emission wavelength of 525 nm (slit: 1.0 nm Bandpass).

2 mL of a solution of calcein-loaded liposomes (diluted 40 times with buffer B) was placed in a quartz cuvette under stirring for fluorescence monitoring. A baseline of calcein fluorescence without polymer addition was normalized for each sample. 20 µL of polymer solutions (in buffer B) of various concentrations were added to the cuvette 50 seconds after the start of the run. After 700 seconds, 20 µL of Triton X (20%) in buffer B was added to completely disrupt the liposomes and therefore to determine the fluorescence intensity corresponding to 100% dye leakage. The measured fluorescence intensity was normalized into percentage leakage activity, Y, using Equation 1:

\[
Y = \frac{I_t - I_{t=0}}{I_{t=0}} \times 100
\]

Iₜ is the fluorescence intensity at time t before the addition of the polymer samples, and I₀ is the Iₜ after the addition of Triton-X.

To determine the 50% polymer-induced dye leakage, EC₅₀, the leakage percentage just before the addition of Triton-X was plotted versus the final polymer concentration (in 2 mL samples) in a semi-logarithmic plot.

**Dynamic light scattering measurements**

Kinetics measurements were performed on a DLS (Zetasizer Ultra, Malvern Panalytical, United Kingdom) at a measurement angle of 173° and 25 °C. 1 mL of liposomes suspension (40 times diluted in buffer B) was placed in a disposable 4 mL polystyrene cuvette. 10 µL of the polymer was added to the cuvette directly before the start of the measurement. Intensity fluctuations were recorded over 1 minute and the corresponding decay rate was calculated by fitting the correlation functions with the cumulant method. The hydrodynamic radius is calculated from Z-average diffusion for 10 measurements points in a time series. A spectroLight 610 (Xtal-concept, Germany) with a 96 well plate reader was also used to determine the radius distribution of aggregation of liposomes after 10 min of incubation with polymer at 23 °C.

**Quartz Crystal Microbalance with Dissipation (QCM-D)**

Measurements were performed on a four-chamber Q-Sense E4 system (Biolin Scientific) using silicon oxide sensors crystals (QSX 303). To monitor the adsorption, frequency (Δf) and dissipation (ΔD) shift data were collected from 4 overtones (3f₀, 5f₀, 7f₀, and 9f₀). Prior to measurements, the crystals were successively cleaned in acetone, methanol, milliQ water, then air-dried, and finally, plasma cleaned. The cleaned crystals were directly placed in the QCM-D chambers.
and resonance frequency was determined first in the air, then degassed PBS buffer was injected in at a flow rate of 50 µL min⁻¹ using an IPC peristaltic pump up to when a constant baseline of \( f_0 \) and \( \Delta f \) was obtained. The phosphate buffer saline (PBS) was prepared by dissolving NaCl (8.00 g, 137 mmol), KCl (0.20 g, 2.7 mmol), Na2HPO4 (1.42g, 10 mmol), and KH2PO4 (0.24 g, 1.8 mmol) in milliQ water (1 L). Then liposomes solutions (1 mg mL⁻¹) were injected to form the lipid bilayer on the SiO₂ crystals. For this, RBC and E. coli-liposomes made of DOPC-DOPS (90% - 10%) and POPE-POPG (80% - 20%), respectively, were used. The flow of liposomes was maintained until stable signals of \( f_0 \) and \( \Delta f \) were obtained. The lipids in excess were then rinsed with the medium PBS before injection of polymer solutions at a concentration of 156 µg mL⁻¹ dissolved in PBS. Finally, the crystals were rinsed by a consecutive flow of PBS and milliQ water. To prevent the formation of air bubbles in the tubing system, the flow rate was interrupted shortly each time the solutions were changed. The \( f_0 \) and \( \Delta f \) steady-state values from the 5th overtone were chosen to determine the mass of polymers adsorbed on the supported lipid layers using the following modified Sauerbrey equation (Equation 2)\[m = \frac{C}{\pi} \left( \Delta f + \frac{\Delta \Delta f}{2} \right),\]\(\text{Equation 2}\) where \( f_0 \) represents the resonance frequency of the crystal (\( f_0 = 4.95\text{MHz}), C \) for the crystal constant (\( C = 17.7\text{ng cm}²\)), \( n \) is the overtone number (\( n = 5\)), and \( m/A \) the adsorbed mass normalized against the apparent area. The molecular weights obtained by aqueous SEC were incorporated in Equation 2 for the chain density calculations. The time-resolved adsorption/desorption of the polymers was approximated with a simple exponential decay function:
\[m/A(t) = m_{eq} - \frac{m_{eq} - m_0}{e^{-\left(\frac{t-t_0}{\tau}\right)}},\]\(\text{Equation 3}\) with \( m/A \) (t) at any time \( t \), \( m_{eq}/A \) as adsorbed mass in equilibrium, \( \Delta m/A \) as adsorbed mass difference from initial to equilibrated state, \( \Delta m = m_{eq} - m_0 \). Fitting the data was done by the Origin software using the Quick fit exponential decay function \( y = y_0 + A1\exp(-t(x0)/t1) \). Cryo-EM images were collected under low-dose conditions on a Thermo Fisher Talos F200C microscope operated at 200 kV equipped with a 4 x 4 Keta 16 M CMOS camera. Thermo Fishers Velox software was used for imaging. In total 20 images were recorded for each condition at a magnification of 13500x resulting in a pixel size of 10.68 Å.

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**References**
