

## “Doubly Selective” Antimicrobial Polymers: How Do They Differentiate between Bacteria?

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**Abstract:** We have investigated how doubly selective synthetic mimics of antimicrobial peptides (SMAMPs), which can differentiate not only between bacteria and mammalian cells, but also between Gram-negative and Gram-positive bacteria, make the latter distinction. By dye-leakage experiments on model vesicles and complementary experiments on bacteria, we were able to relate the Gram selectivity

to structural differences of these bacteria types. We showed that the double membrane of *E. coli* rather than the difference in lipid composition between *E. coli* and *S. aureus* was re-

sponsible for Gram selectivity. The molecular-weight-dependent antimicrobial activity of the SMAMPs was shown to be a sieving effect: while the 3000 gmol<sup>-1</sup> SMAMP was able to penetrate the peptidoglycan layer of the Gram-positive *S. aureus* bacteria, the 50000 gmol<sup>-1</sup> SMAMP got stuck and consequently did not have antimicrobial activity.

**Keywords:** antibacterial polymers • biological activity • ring-opening polymerization • peptidomimetics • vesicles

### Introduction

Due to the epidemic spread of multiple antibiotic resistant bacteria in hospitals<sup>[1]</sup> and other public institutions, synthetic mimics of antimicrobial peptides (SMAMPs) are becoming increasingly important for a wide range of applications, including medical devices and healthcare products. SMAMPs are polymers designed to capture the essential features of antimicrobial peptides (AMPs). They contain cationic hydrophilic groups that allow them to attach to the negatively charged bacterial cell membrane.<sup>[2]</sup> Hydrophobic moieties then trigger membrane permeation and disruption of the pathogen cells, leading to a breakdown of the membrane potential, plasma leakage, and cell death.<sup>[3]</sup> Their activity is usually not targeted against specific structures such as receptors, which is why AMPs are a crucial component of the innate immune system, and lead to less resistance formation

than conventional antibiotics.<sup>[4]</sup> The desirable properties of SMAMPs are a high antibacterial activity (a low MIC<sub>90</sub> value<sup>1</sup>) and low red blood cell lysis (a high HC<sub>50</sub> value<sup>2</sup>), leading to a high selectivity<sup>3</sup> for bacteria over the mammalian host cells. SMAMPs that are equally active against Gram-positive bacteria (e.g., *Staphylococcus aureus*) and Gram-negative bacteria (e.g., *Escherichia coli*) are well described in the literature.<sup>[5-8]</sup> Recently, we reported antimicrobial polymers with an astonishing “double selectivity”: first, for bacteria over mammalian cells, and second, for Gram-positive over Gram-negative bacteria.<sup>[9]</sup> Gram selectivity is rare in the SMAMP literature. Recently, Kallenbach et al. reported a synthetic peptide with a selectivity of 4 for *E. coli* over *S. aureus* (which compares to the natural AMP gentamicin with a selectivity of about 40).<sup>[10]</sup> Muehle and Tam reported a design concept for the synthesis of AMPs with Gram-negative selectivity through binding to the lipopolysaccharide layer of Gram-negative bacteria.<sup>[11]</sup> SMAMPs with Gram selectivity for Gram-positive bacteria

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Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/chem.200802558>.

<sup>1</sup> MIC<sub>90</sub> = minimum inhibitory concentration that will reduce the growth of a certain bacteria by 90% as compared to an untreated control. Low MIC<sub>90</sub> values indicate a high antibacterial activity.

<sup>2</sup> HC<sub>50</sub> = hemolytic concentration at which 50% of human red blood cells are lysed. Samples that yield high HC<sub>50</sub> values have a low toxicity.

<sup>3</sup> Selectivity = HC<sub>50</sub>/MIC<sub>90</sub>: high selectivity values imply a good tolerance by the host organism, combined with a high toxicity towards the target organism.

have, to the best of our knowledge, not yet been reported, and the questions we address in this work concern the molecular origin of this selectivity.

The morphological differences between Gram-negative and Gram-positive cells are significant: The Gram-negative cell (Figure 1 a) has an outer and a plasma membrane, with

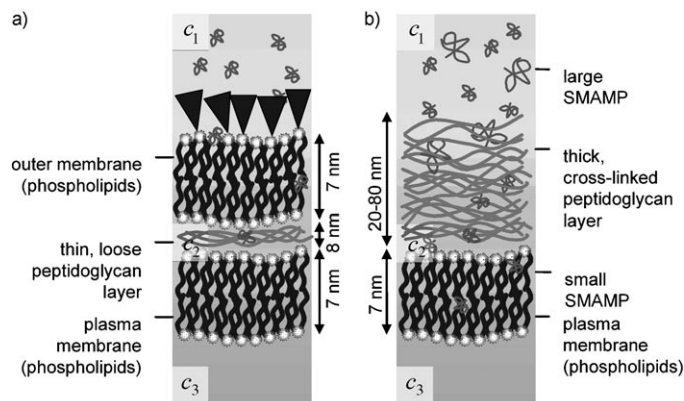


Figure 1. Cartoon representation of the cell membrane and cell-wall morphology of a) *E. coli* (Gram-negative) and b) *S. aureus* (Gram-positive), and their interactions with SMAMPs; details like ion channels, and membrane proteins are omitted for clarity.

a thin layer of loosely cross-linked peptidoglycan in the periplasmic space between the two lipid membranes, and a thick layer of lipopolysaccharide (LPS) on the outer membrane. On the other hand, the Gram-positive bacteria cell (Figure 1 b) has a 20–80 nm thick, highly cross-linked peptidoglycan layer surrounding a single bilayer lipid membrane. Additionally, the membranes consist of different phospholipids: Generally, Gram-negative bacteria (and specifically *E. coli*) membranes mainly consists of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), while the membrane of *S. aureus*, like most other Gram-positive bacteria, is made predominantly from cardiolipin (CL).<sup>[12,13]</sup>

We set out with the hypothesis that the molecular origin for the observed double selectivity could be the general structural difference, specifically the different lipid composition, of the plasma membranes. We further postulate that the additional peptidoglycan layer of *S. aureus* might be responsible for a molecular-weight dependence seen in many previously reported SMAMPs, namely that antimicrobial activity is lost with increasing molecular weight. Using dye-leakage experiments on bacteria-mimicking model vesicles—and complementary cell studies—we can relate the observed double selectivity of a model SMAMP<sup>[14]</sup> (Figure 2 a) to the morphological differences between these two bacterial types.

## Results and Discussion

The SMAMP that was used as a probe to test the above described hypotheses is shown in Figure 2 a. It was obtained by

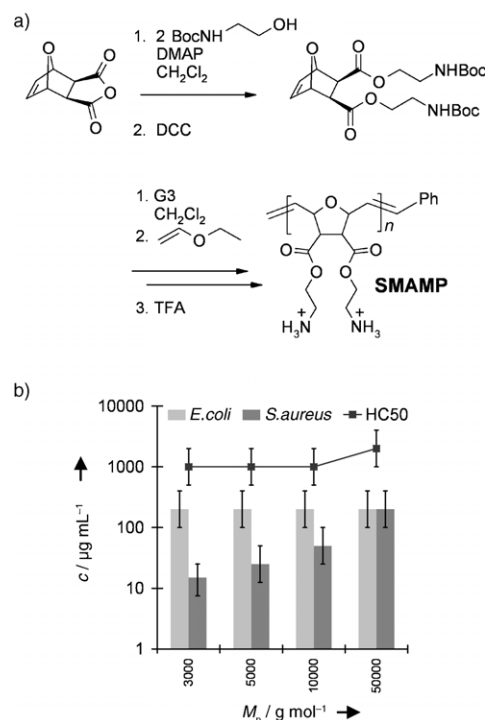


Figure 2. a) Structure and synthesis of the SMAMP. b) SMAMP antimicrobial and hemolytic properties (Grey bars: minimum inhibitory concentration (MIC<sub>90</sub>), which is the SMAMP concentration that inhibits the bacterial growth by 90%, against *E. coli* (light grey) and *S. aureus* (dark grey); Black squares: hemolytic concentration (HC<sub>50</sub>), which is the SMAMP concentration that lyses 50% of human red blood cells). MIC<sub>90</sub>s and HC<sub>50</sub> are plotted against SMAMP molecular weight.

ring-opening metathesis polymerization (ROMP) and deprotection from an oxanorbornene monomer carrying two Boc-protected amine groups. By varying the monomer to initiator ratio, different molecular weights were obtained. Synthesis details for this compound are given elsewhere.<sup>[14]</sup> The biological data (HC<sub>50</sub> and MIC<sub>90</sub>) for the different molecular weights (3000–50000 g mol<sup>-1</sup>) is summarized in Figure 2 b, which shows that the 3000 g mol<sup>-1</sup> sample is doubly selective (Selectivity = 67 for *S. aureus* over human erythrocytes, and 13 for *S. aureus* over *E. coli*) and that the SMAMP has a molecular-weight-dependent antimicrobial activity against *S. aureus*, ranging from an MIC<sub>90</sub> of 15 μg mL<sup>-1</sup> for the 3000 g mol<sup>-1</sup> sample, to an MIC<sub>90</sub> of 200 μg mL<sup>-1</sup> for the 50000 g mol<sup>-1</sup> sample.

Since it is generally accepted that SMAMPs, like AMPs, interact with bacteria in such a way that their membranes are corrupted (although other cell targets can exist),<sup>[15]</sup> we used dye-leakage experiments on unilamellar vesicles as a model system to probe the interaction of our model SMAMP with membranes.<sup>[13]</sup> The principle of this experiment is illustrated in Figure SI1a in the Supporting Information.

In a first series of experiments, the model vesicles for *E. coli* bacteria (consisting of PE/PG lipid) and *S. aureus* bacteria (made from CL lipid) were exposed to the four SMAMPs with molecular weights from 3000 to

50 000  $\text{g mol}^{-1}$  at a single concentration. The dye-leakage data obtained showed that the SMAMPs, regardless of their molecular weight, were membrane active against both model membranes at all molecular weights (Figure SI 1b and SI 1c in the Supporting Information). As dye-leakage data obtained at a single polymer concentration are not necessarily the most quantitative measure of membrane activity, a concentration-dependent leakage curve was obtained for the 3000  $\text{g mol}^{-1}$  SMAMP (Figure 3 a). Fitting this data to

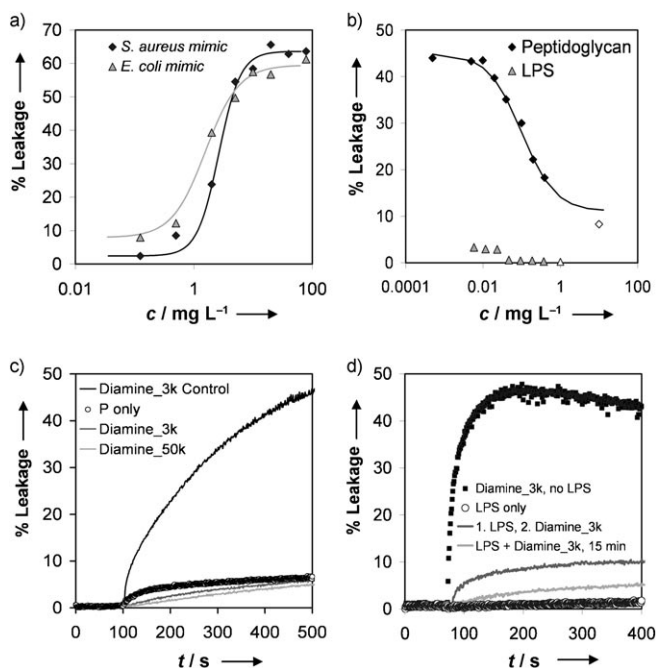


Figure 3. Dye-leakage experiments on model membranes: a)  $EC_{50}$  curves for the 3000  $\text{g mol}^{-1}$  SMAMP (*E. coli* mimics and *S. aureus* mimics); b) effect of increasing peptidoglycan concentration (solid diamonds) and increasing LPS concentration (solid triangles) at constant polymer concentration; open diamond: LPS only, open triangle: peptidoglycan only; c) membrane-active SMAMP in the presence of peptidoglycan: Leakage is significantly reduced as compared to the control sample; d) membrane-active SMAMP in the presence of LPS—after sufficient incubation time (24 hours), leakage is drastically reduced.

the Hill equation<sup>[13]</sup> gave the polymer concentration that leads to 50% polymer-induced leakage,  $EC_{50}$ . For the PE/PG vesicles,  $EC_{50}$  was 1.57  $\mu\text{g mL}^{-1}$  and for the CL vesicles 2.64  $\mu\text{g mL}^{-1}$ ; additionally, both vesicle types have the same maximum leakage. Thus, the 3000  $\text{g mol}^{-1}$  SMAMP is equally membrane-active against *S. aureus* and *E. coli* mimics. The data shows that this simple model system failed to emulate the mechanisms that cause Gram selectivity and molecular-weight selectivity; however we can conclude from these experiments that the different lipid composition of *E. coli* and *S. aureus* is not responsible for the observed differences in antimicrobial activity, specifically the inactivity against *E. coli*.

Although vesicle studies are common in the (SM)AMP literature, these unilamellar vesicles are understood to be extremely simple models of the plasma membrane. They

might be a reasonable model for *S. aureus*, with only one membrane, but do not capture the double membrane structure of *E. coli*. This additional membrane effectively creates a gradient in SMAMP concentration (Figure 1 a). In an MIC experiment, the outer membrane sees a concentration  $c_1$  that causes membrane disintegration; however the periplasmic space sees a significantly reduced concentration  $c_2$ , which is insufficient to damage the plasma membrane. As our experiments proved that the different lipids are not responsible for the observed double selectivity, this led to the assumption that the double-membrane structure might be responsible for the inactivity of the SMAMP against *E. coli*. This idea is consistent with previous observations demonstrating that the mere loss of the outer membrane integrity was not sufficient to kill *E. coli* bacteria.<sup>[16]</sup> There are two alternatives to test this assumption. We considered repeating the dye-leakage experiment with bilamellar vesicles; however, this proved difficult to realize experimentally. Instead, we modified *E. coli* bacteria in such a way that their outer membrane was damaged and thereby obtained a model for a Gram-negative cell with only one intact membrane. This approach involves perforating *E. coli* cells with EDTA for one minute, followed by quenching with  $\text{CaCl}_2$ , and is well-documented in the literature (see the Supporting Information for details).<sup>[17–19]</sup> We exposed these membrane-compromised *E. coli* bacteria and regular *E. coli* cells to the 3000  $\text{g mol}^{-1}$  SMAMP and compared the MIC curves obtained. It was found that the EDTA-treated *E. coli* cells are susceptible to the SMAMP ( $\text{MIC}_{90} = 100 \mu\text{g mL}^{-1}$ ), while the SMAMP remained inactive against regular *E. coli* cells ( $\text{MIC} > 200 \mu\text{g mL}^{-1}$ ) (Figure SI2a in the Supporting Information). We thus demonstrated that the Gram selectivity of the 3000  $\text{g mol}^{-1}$  SMAMP is a result of the double membrane structure of *E. coli*. The question that remains unanswered at this point is whether the observed effect is due to the actual second lipid layer, or the LPS layer of the outer membrane. To investigate this in more detail, MIC testing on *S. aureus*, which has only one lipid membrane, was performed in the presence of LPS extract from *E. coli* (Figure SI4 in the Supporting Information). The data showed that LPS itself is not toxic ( $\text{MIC}_{90} > 200 \mu\text{g mL}^{-1}$ ) to *S. aureus*, and that at LPS concentrations up to 67  $\mu\text{g mL}^{-1}$ , the  $\text{MIC}_{90}$  of the 3000  $\text{g mol}^{-1}$  SMAMP remains unchanged. At 133  $\mu\text{g mL}^{-1}$  LPS, the  $\text{MIC}_{90}$  increases slightly, possible due to increasing SMAMP–LPS binding at such a high LPS concentration (LPS/SMAMP  $\approx 10:1$ ). According to the literature,  $10^8$  bacteria cells contain 0.5  $\mu\text{M}$  LPS, thus the LPS concentration in a regular MIC experiment is  $\approx 5 \mu\text{g mL}^{-1}$ .<sup>[20]</sup> This is significantly lower than the amount of LPS that was added to *S. aureus*. Thus, SMAMP binding to LPS in this concentration range does not decrease SMAMP activity.

In a second series of experiments, we tested the hypothesis that the peptidoglycan layer around the outer membrane of *S. aureus* causes the observed loss of antimicrobial activity with increasing molecular weight. According to the dye-leakage data for the *S. aureus*-like vesicles (Figure SI 1c), in

the absence of the peptidoglycan layer, all SMAMPs were membrane-active regardless of their molecular weight  $M_n$ . Based on these results, we propose that the higher  $M_n$  SMAMPs cannot reach the plasma membrane of *S. aureus*, because they cannot pass the highly cross-linked peptidoglycan layer (Figure 1b), which effectively reduces their concentration  $c_2$  at the plasma membrane and renders them inactive in the MIC<sub>90</sub> experiments. The peptidoglycan layer consists of anionically charged, alternating copolymers of  $\beta$ -(1,4)-linked *N*-acetylmuramic acid and *N*-glucosamine, which are cross-linked by peptide chains. There are two possible  $M_n$ -dependent modes of interaction between this layer and SMAMPs: First, there could be SMAMP-peptidoglycan binding: Whatever the nature of this binding, whether it is charge-, polarity- or hydrophobicity-driven, the number of binding sites per chain will increase with  $M_n$ , making the binding gradually more irreversible. Second, the peptidoglycan might simply act as a sieve, thus, the higher the  $M_n$  of the SMAMP and the larger its hydrodynamic volume, the less likely it is to pass through the peptidoglycan mesh.

As a mimic of this highly cross-linked peptidoglycan layer, we used peptidoglycan extract from *S. aureus* bacteria in the dye-leakage experiments. While this extract does not form a dense, uniform layer around the vesicle when added to a dye-leakage experiment and thus is not a perfect model for the sieving properties of the peptidoglycan layer of a real *S. aureus* cell, it is a model to investigate SMAMP-peptidoglycan binding.

The following experiments were designed to investigate the effect of peptidoglycan, and to differentiate between the two proposed modes of SMAMP-peptidoglycan interaction.

- 1) CL vesicles were incubated with the peptidoglycan extract for 10 min and 24 h, after which times the vesicles were exposed to the SMAMP. We found that, while the extract itself did not cause leakage, all molecular weights of the SMAMP were membrane-active in the presence of peptidoglycan for both incubation times.
- 2) CL vesicles were exposed to samples containing constant concentrations of the 3000 g mol<sup>-1</sup> SMAMP, which were previously incubated for 24 h with different amounts of peptidoglycan extract. Plotting the concentration of the peptidoglycan versus the induced maximum leakage percentage, a peptidoglycan concentration-dependent curve was obtained (Figure 3b). These data show that the polymer-peptidoglycan ratio is critical for the amount of leakage reduction. At a sufficiently high peptidoglycan concentration, the leakage was near-quantitatively quenched, that is, saturation was reached.
- 3) CL vesicles were mixed with SMAMP solutions that had been incubated with the previously determined saturation amount of peptidoglycan for 10 min and 24 h. In the case of the short incubation time, all polymers were membrane active in the presence of the extract, meaning that no SMAMP-peptidoglycan complex formed. However after 24 h, as shown in Figure 3c, the membrane activity of all samples was lost, indicating binding.

The results of these experiments are illustrated in Figure 4. We interpret the data as follows: SMAMP-peptidoglycan binding does occur, but it is a relatively slow pro-

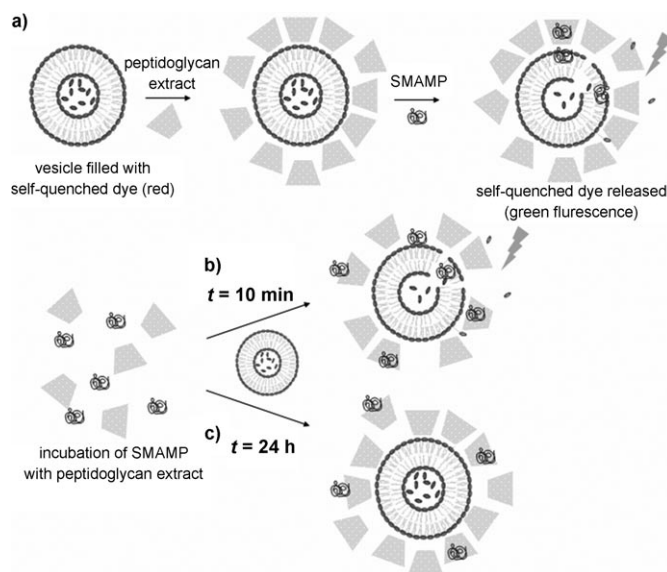


Figure 4. Rationalization of the results of the dye-leakage experiments in the presence of peptidoglycan: a) when first adding the peptidoglycan extract, and then the SMAMP, membrane activity is observed; b) when incubating the SMAMP with the peptidoglycan extract for 10 min, the polymers are still membrane active; c) incubation for 24 h quenches the membrane activity.

cess. Thus, on the timescale of an MIC<sub>90</sub> experiment, the loss of SMAMP activity with increasing  $M_n$  is not caused by binding. As predicted, the CL model vesicles with non-cross-linked peptidoglycan are not able to differentiate between different  $M_n$  polymers, because the added peptidoglycan does not form a uniform layer around the vesicle, which is why both the high and the low  $M_n$  SMAMP are active when only incubated for 10 min. In the parent bacteria, however, the peptidoglycan layer is thick and uniform, and the pores have a finite size, meaning that only smaller molecules can diffuse through easily (Figure 1b). Thus, the high-molecular-weight SMAMPs could only pass this layer by a reptation-like mode, meaning that the polymer coil would have to unravel. This process is so slow that, on the timescale of the MIC experiment, no antimicrobial activity is observed. This is consistent with the observation that only proteins with a diameter of up to 2 nm were able to pass the cross-linked peptidoglycan layers that had been isolated from *E. coli* and *B. subtilis*.<sup>[21]</sup>

Out of curiosity, we repeated these binding experiments with vesicles that mimic *E. coli* (PE/PG) and LPS. The results are shown in Figure 3b and 3d. We first added LPS to the vesicles, which caused no leakage. We then added LPS, let it incubate with the vesicles for 1 h, and added the 3000 g mol<sup>-1</sup> SMAMP. The leakage obtained compared to the control with no LPS was considerably reduced (Figure 3d). However, when LPS was added to the vesicle, fol-

lowed by SMAMP addition after only 2 min, the SMAMP activity was not significantly quenched (not shown). Thus, the interaction of LPS with the vesicle is time-dependent and only after sufficient incubation is a reduction of leakage observed upon SMAMP addition. In another experiment, we incubated LPS with the SMAMP for 15 min and added the mixture to the vesicle. The leakage was drastically reduced due to SMAMP–LPS binding (Figure 3d). To directly compare the strength of binding of our model SMAMP with peptidoglycan and LPS, respectively, we incubated the SMAMP with different LPS concentrations over night and obtained a LPS concentration-dependent dye-leakage curve. (Figure 3b). It is clear from this data that at the same concentration, LPS reduces the leakage significantly more than peptidoglycan. From the above body of data, we conclude that the SMAMP–LPS binding is faster and stronger than the SMAMP–peptidoglycan binding. This leads to the interpretation that binding does not play a role for the case of peptidoglycan and the SMAMP, as it is both comparatively weak and too slow on the timescale of the MIC experiment. Thus, the molecular-weight selectivity of the SMAMP versus *S. aureus* is due to sieving of the large molecules by the peptidoglycan layer. For LPS and *E. coli*, the case is less clear cut. On the one hand, SMAMP–LPS binding is strong and fast, as leakage quenching in less than 15 min was observed; on the other hand, in the MIC experiment, the 3000 gmol<sup>-1</sup> SMAMP was active against both membrane-compromised *E. coli* cells and regular *S. aureus* cells with LPS present. This observation favors the interpretation that the Gram selectivity of the SMAMP is due to the double membrane present in *E. coli* (which creates the before mentioned SMAMP concentration gradient) rather than simple LPS binding, although SMAMP–LPS binding, when LPS is in the form of a membrane, may contribute to the effect.

## Conclusions

In conclusion, we were able to give some insight into the molecular origins of the Gram selectivity observed for ROMP-based polymeric SMAMPs. It was shown by dye-leakage experiments on model vesicles that the different lipid composition of Gram-negative versus Gram-positive cells is not responsible for the observed double selectivity. MIC experiments on *S. aureus* in the presence of LPS demonstrated that the SMAMP is not deactivated by binding to lipopolysaccharide, and that damaging the outer membrane of *E. coli* with ETDA makes *E. coli* susceptible to the previously inactive SMAMP. Thus, the selectivity of this SMAMP for Gram-positive over Gram-negative bacteria is most likely due to the double-membrane structure of Gram-negative bacteria, specifically *E. coli*, which efficiently prevents the SMAMP from reaching the plasma membrane in the necessary concentration.

It was further demonstrated that the molecular-weight dependence of the MIC<sub>90</sub> data for Gram-positive bacteria like *S. aureus* is not due to binding to the peptidoglycan layer, but probably a sieving effect, as the highly cross-linked peptidoglycan mesh shields the plasma membrane from large membrane-active molecules and thus renders them membrane-inactive.

## Experimental Section

All experimental procedures, including polymer and monomer synthesis, microbiological assays and dye-leakage experiments, can be found in the Supporting Information.

## Acknowledgements

This work was supported by the German Research Foundation (DFG-Forschungstipendium to K.L.), ONR, MRSEC, NSF and NIH.

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Received: December 5, 2008

Revised: July 22, 2009

Published online: September 29, 2009