Supporting Information

Toll-Like Receptor Agonist Lipopeptides Self-Assemble into Distinct Nanostructures

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Experimental Section

Materials. The structures of the lipopeptides studied are shown in Fig.1. PAM2CSK4 and PAM3CSK4 were supplied as lyophilized powders by Invivogen. This sample of PAM3CSK4 is supplied as a HCl salt. PAMCSK4 and PAM3CSK4 as a TFA salt were purchased from EMC Microcollections (Tübingen, Germany).

The lipopeptides were dissolved in limulus amebocyte lysate (LAL) buffer to give 0.5 wt% solutions. This buffer was used since it is a standard used in bacterial endotoxin assays as commonly performed with these lipopeptides, and as provided by the supplier with the lyophilized lipopeptide powder. This buffer contains an extract from horseshoe crab blood cells, and as such contains a mixture of different ionic species.

Circular Dichroism (CD). CD spectra were recorded using a Chirascan spectropolarimeter (Applied Photophysics, UK). Each sample (0.5 wt% lipopeptide) was placed in a cover slip cuvette (0.1 mm thick). Spectra are presented with absorbance \( A < 2 \) at any measured point with a 0.5 nm step, 1 nm bandwidth, and 1 second collection time per step in the range 20 °C - 60 °C with 10 °C steps on heating and 5 min equilibration at each temperature. Samples were then cooled to 20 °C and after a further 5 min equilibration, spectra were measured. The CD signal from the LAL water was subtracted from the CD data of the PAM solutions.
Small-Angle X-ray Scattering (SAXS). Experiments were performed on beamline BM29 at the ESRF (Grenoble, France). A few microlitres of samples were injected via an automated sample exchanger at a slow and very reproducible flux into a quartz capillary (1.8 mm internal diameter), which was then placed in front of the X-ray beam. The quartz capillary was enclosed in a vacuum chamber, in order to avoid parasitic scattering. After the sample was injected in the capillary and reached the X-ray beam, the flow was stopped during the SAXS data acquisition. The sample was thermostated throughout its entire travel from the injector to the quartz capillary. SAXS experiments were performed at 20 °C. The $q = 4\pi \sin(\theta)/\lambda$ range is approximately 0.04-5 nm$^{-1}$, with $\lambda = 1.03$ Å (12 keV) and a 2.87 m sample-detector distance. The images were captured using a PILATUS 1M detector. Data processing (background subtraction, radial averaging) was performed using dedicated beamline software ISPYB.

Cryo-Transmission electron microscopy (cryo-TEM). Experiments were carried out using a field emission cryo-electron microscope (JEOL JEM-3200FSC) operating at 200 kV. Images were taken using bright-field mode and zero loss energy filtering (omega type) with a slit with 20 eV. Micrographs were recorded using a Gatan Ultrascan 4000 CCD camera. The specimen temperature was maintained at -187 °C during the imaging. Vitrified specimens were prepared using an automated FEI Vitrobot device using Quantifoil 3.5/1 holey carbon copper grids with 3.5 μm hole sizes. Grids were cleaned using a Gatan Solarus 9500 plasma cleaner just prior to use and then transferred into an environmental chamber of FEI Vitrobot at room temperature and 100% humidity. Thereafter, 3 μl of sample solution at 2 wt%
concentration was applied on the grid, blotted once for 1 second and then vitrified in a 1/1 mixture of liquid ethane and propane at -180 °C. Grids with vitrified sample solutions were maintained in a liquid nitrogen atmosphere and then cryo-transferred into the microscope.
SI Table 1. SAXS form factor model fitting parameters. The modelling was done using the freely available software SASfit. The corresponding equations can be found in the online SASfit manual.

### Spherical Micelle Form Factor

<table>
<thead>
<tr>
<th>Sample</th>
<th>R(outer)(^a)/nm</th>
<th>R(inner)(^b)/nm</th>
<th>(\mu^c)</th>
<th>(\nu^d)</th>
<th>Gaussian polydispersity parameters [R(outer)](^e)</th>
<th>Background(^f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAMCSK4</td>
<td>2.23</td>
<td>1.63</td>
<td>-0.42</td>
<td>0.69</td>
<td>N=1, (\sigma=0.625)</td>
<td>10</td>
</tr>
<tr>
<td>PAM2CSK4</td>
<td>3.30</td>
<td>2.42</td>
<td>-0.67</td>
<td>0.033</td>
<td>N=1, (\sigma=0.54)</td>
<td>0.08</td>
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</tbody>
</table>

### Gaussian Bilayer Form Factor

<table>
<thead>
<tr>
<th>Sample</th>
<th>(t^g)/nm</th>
<th>(\sigma)(core), (\sigma)(out)(^h)/nm</th>
<th>(\nu)(core)(^i)</th>
<th>(\nu)(out)(^j)</th>
<th>Gaussian polydispersity parameters (t)(^k)</th>
<th>Background(^l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAM3CSK4 (TFA salt)</td>
<td>5.33</td>
<td>3.89, 0.82</td>
<td>-0.004</td>
<td>0.02</td>
<td>N=0.17, (\sigma=0.71)</td>
<td>5.19</td>
</tr>
</tbody>
</table>

\(a\) Radius of outer core of micelle  
\(b\) Radius of inner core of micelle  
\(c\) Ratio of core/shell scattering contrasts  
\(d\) Shell/solvent scattering contrast (arbitrary units)  
\(e\) Gaussian peak height N and half-width \(\sigma\)  
\(f\) Constant background term  

\(g\) Bilayer thickness  
\(h\) Width for core (lipid chain) electron density, \(\sigma\)\(_{\text{core}}\), and outer (peptide) electron density, \(\sigma\)\(_{\text{out}}\). Gaussian functions  
\(i\) Scattering contrast of lipid core of bilayer (with respect to solvent) (arbit. units)  
\(j\) Scattering contrast of peptide units at bilayer surface (with respect to solvent) (arbit. units)  
\(k\) Gaussian peak height N and half-width \(\sigma\)  
\(l\) As well as the constant background, the radius of the bilayer objects was fixed at 500 nm. Since this is much larger than \(t\) it does not influence the shape of the scattering profile.
**Fig. S1.** Cryo-TEM images obtained for PAM3CSK4(TFA). To be compared to image for PAM3CSK4(HCl) shown in Fig. 1c.

**Fig. S2.** SAXS data for PAM3CSK4 salts (top) TFA, (bottom) HCl.