

Supporting Information for

A short non-cytotoxic antimicrobial peptide designed from A β ₂₉₋₄₀ adopts nanostructure and shows *in vivo* anti-endotoxin activity

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Experimental Section

Materials

Rink amide MBHA resin (loading capacity: 0.36-0.78 mmol/g), N- α -Fmoc and necessary side-chain protected amino acids were purchased from Novabiochem. Coupling reagents for peptide synthesis including Oxyma Pure [ethyl 2-cyano-2-(hydroxyimino) acetate] and HCTU [O-(6-chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate] and were purchased from Novabiochem, DIC (N,N'-diisopropylcarbodiimide), DIPEA (N,N-diisopropylethylamine) and NMM (N-methylmorpholine) were purchased from Sigma, USA. Dichloromethane (DCM), N,N-dimethylformamide (DMF), piperidine, diethyl ether and trifluoroacetic acid (TFA) were of standard grades and procured from reputed local companies. Ninhydrin, KCN, valinomycin and cholesterol were purchased from Sigma. Egg phosphatidylcholine (PC) and egg phosphatidylglycerol (PG) were obtained from Avanti Polar

Lipids, Inc., USA whereas 3,3'-dipropylthiadicarbocyanine iodide (diS-C₃-5), 5(6)-carboxytetramethylrhodamine N-succinimidyl ester [5(6)-TAMRA, SE], propidium iodide (PI) and Alexa Fluor-annexin V were purchased from Invitrogen, USA. *E. coli* 0111:B4 lipopolysaccharide and FITC-LPS *E. coli* 0111:B4 from Sigma. For cell culture, RPMI 1640, Fetal Bovine Serum, 100X Antibiotic-antimycotic and 0.25% Trypsin-EDTA (1X) were purchased from Gibco/Invitrogen. Rests of the reagents are of analytical grade and were procured locally; buffers were prepared in Milli Q water (USF-ELGA).

Cytokine estimation kits

Human TNF- α ELISA Set, Human IL-1 β ELISA Set, Mouse TNF- α ELISA Set II, Mouse IL-6 ELISA Set and TMB Substrate Reagent Set were purchased from BD Biosciences.

Bacterial strains, cell lines and animals

The bacterial strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC BAA-427, *Klebsiella pneumoniae* ATCC 27736, *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633 were obtained from the American Type Culture Collection (ATCC). THP-1 and 3T3 cell lines were obtained from CSIR-Central Drug Research Institute (CSIR-CDRI), Lucknow cell line repository. The cell lines were maintained by the usual protocol in an Innova CO₂ incubator. Animals for experiments were provided by National Laboratory Animal Center, CSIR-CDRI. All animal procedures were carried out according to the protocols approved by the Institutional Animal Ethics Committee (No. IAEC/2015/90) and National Laboratory Animal Centre.

Methods

Peptide synthesis, fluorescent labeling and purification

All the peptides were synthesized with a peptide synthesizer (PS3 model, Protein Technologies, Inc.) using 9-fluorenylmethoxycarbonyl (Fmoc) solid phase method on rink amide 4-methylbenzhydrylamine (MBHA) resin as described previously^{1, 2}. Labeling at the N-termini of peptides with a fluorescent probe was achieved by a standard procedure². Briefly, Fmoc deprotected resin-bound peptides were incubated with vigorous shaking to 5(6)-carboxytetramethylrhodamine N-succinimidyl ester (5(6)-TAMRA, SE) (2-3 equiv.) in DMF in the presence of 5% DIPEA for 48-72 h, which ultimately resulted in the formation of N ^{α} -Rho-peptides. The peptides were cleaved from the resin and precipitated with dry ether. All the

peptides were purified by RP-HPLC on a semi-preparative Waters C18 column using a linear gradient of 10–90% acetonitrile in 40 min with a flow rate of 2.0 ml/min and each peptide was subjected to MALDI-TOF analysis for the detection of their molecular masses.

Hemolytic activity assay of the peptides

Hemolytic activities of the peptides were determined as reported previously². Briefly, fresh human red blood cells (hRBCs) were collected in the presence of an anticoagulant from a healthy volunteer. Our experimental protocol with human blood was as per the guidelines and regulations of CSIR-Central Drug Research Institute Ethics Committee and was approved by it with approval No. CDRI/IEC/2014/A5. Moreover, informed consent was obtained from the healthy volunteer before collection of blood in accordance with the guideline of our Institutional ethics committee. Fresh hRBCs were washed with PBS until the supernatant was clear. Hemolytic activity of the peptides was determined against 4% (v/v) of the human red blood cells (hRBCs) in PBS by assaying the ability of the peptides to lyse the hRBCs. The antimicrobial peptide solution (100 µl) in PBS at various concentrations up to 100 µM were placed into microcentrifuge tubes and mixed with 100 µl of the red blood cell suspension. These mixtures were then incubated at 37 °C for 45 min in water bath. After 45 min hemoglobin release was measured by checking the UV absorbance of the samples at 540 nm. As a negative and positive control, untreated red blood cell suspension and 5% Triton X-100 containing suspension of red blood cells were used, respectively. Each assay was repeated for 3 times. Percentage of hemolysis was as follows:

$$\% \text{ Hemolysis} = [(A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{triton}} - A_{\text{blank}})] \times 100$$

Cell viability assay

To determine cytotoxicity of the peptides viability of 3T3 cells was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described earlier³.

Antimicrobial activity assay of the peptides

Antimicrobial activity against different Gram-positive and Gram-negative bacterial strains was determined by broth microdilution method⁴. In brief, bacteria were grown in Mueller-Hinton broth to the mid-log phase and 50 µl of diluted bacterial culture (10⁶ CFU/ml) were added to 50 µl of serially diluted different peptides in medium containing 0.02% acetic acid 0.4% BSA in each well of 96-well culture plate and incubated for 16–18 h at 37 °C. Antibacterial activity of

the peptide was expressed as their MICs (the peptide concentration at which ~100% inhibition of microbial growth takes place). The antibacterial activity was carried out thrice independently in triplicate.

To determine how much time a peptide takes to kill a bacterium, we performed bactericidal kinetics assay. Briefly, *E. coli* ATCC 25922 (10^6 CFU/ml) was treated with $5 \times$ MIC of the peptide. Aliquots of 10 μ l were removed at varying time intervals (0, 30, 60, 120, 180 min) and plated on an agar plate. The colonies were counted after incubation for 16-18 h at 37 °C⁵.

Serum stability and salt sensitivity

To examine the effect of serum, antimicrobial activity of the peptides were assayed in 10% FBS (Fetal Bovine Serum) against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. For salt sensitivity, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were incubated in the presence of different concentrations of physiological salts to give the following final concentrations: 150 mM NaCl and 4.5 mM KCl. The MICs of the peptides were determined as described above and the results are shown are from three independent assays.

Assay of peptide-induced depolarization of hRBC and bacterial membrane

Peptide-induced depolarization of the hRBC and bacterial membrane was studied by measuring the efficacy of a peptide to dissipate the potential across these cell membranes. Fresh human red blood cells washed with PBS (as used for hemolytic activity assay which has been described previously) and were resuspended in the same buffer with a final cell volume of 0.6% (v/v)⁶. In case of bacteria, culture was grown to mid-log phase at 37 °C and centrifuged followed by washing with 5 mM HEPES (pH 7.2) containing 20 mM glucose, 100 mM KCl and 0.5 mM EDTA. Then bacteria were resuspended to 5×10^6 CFU/ml in the same buffer. Both hRBCs and bacteria were incubated with diS-C₃-5 dye (final concentration 4 μ M) for 1 h. When the fluorescence level (fluorescence of the dye was monitored at 670 nm with an excitation wavelength of 622 nm) of the hRBCs or bacterial suspension became stable, different concentrations of each of the peptides were added to these suspensions in order to record the peptide-induced membrane depolarization of either hRBCs or bacterial membrane. Membrane depolarization as measured by the fluorescence recovery (F_t) was defined by the equation:

$$F_t = \frac{I_t - I_0}{I_f - I_0} \times 100$$

where I_f , the total fluorescence, was the fluorescence levels of cell suspensions just after addition of diS-C₃-5; I_t was the observed fluorescence after the addition of a peptide at a particular concentration either to hRBCs or to bacterial suspensions, which were already incubated with diS-C₃-5 dye for 1 h and I_0 , was the steady-state fluorescence level of the cell suspensions after 1 h incubation with the dye.

Assay of peptide-induced dissipation of diffusion potential of different lipid vesicles

Peptides-induced permeabilization membrane bilayer was measured by assaying their ability to dissipate the diffusion potential across the zwitterionic mammalian membrane mimetic, PC/Chol (8:1, w/w) and negatively charged bacterial membrane mimetic, PC/PG (3:1 w/w) lipid vesicles that mimic the mammalian and bacterial membrane respectively as described earlier⁷.

Detection of peptide-induced membrane damage of hRBCs and bacterial cells

Peptide-induced phospholipid asymmetry or damage of phospholipid membrane organization of hRBCs was determined by staining the cells ($\sim 5.0 \times 10^6$ cells/ml) with Alexa Fluor-annexin V after the treatment with the peptides at room temperature for 15-20 min⁸. Extent of staining was measured by analyzing peptide treated cells with respect to peptide untreated control using Becton Dickinson FACSCalibur flow cytometer with a laser excitation wavelength of 488 nm.

To check the peptide-induced damage to membrane integrity of *E. coli* ATCC 25922, the cells were grown to mid-log phase, washed with PBS and diluted to $\sim 10^8$ CFU/ml. Now this diluted bacterial culture incubated with peptides for 30 min at 37 °C with constant shaking at 120 rpm. The cells were centrifuged, washed two times with PBS, and incubated further with propidium iodide (PI) at 4 °C for 30 min., followed by removal of the unbound dye through washing with an excess of PBS and resuspended in the same buffer. Peptide-induced damage of bacterial cells was then analyzed by flow cytometer as mentioned above.

Scanning electron microscopy (SEM)

Peptide-induced morphological changes on the cell membrane of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, were studied by employing scanning electron microscopy as described earlier⁷. In brief, mid-log phase grown bacteria ($\sim 10^8$) were treated with different peptides for 1 h. After treatment the cells were washed with 10 mM sodium phosphate buffer (pH 7.4), adhered on poly-L-lysine coated glass chips and fixed with 2% glutaraldehyde and 4% formaldehyde in the same buffer overnight. Then the cells were osmicated and gradually dehydrated in an

ascending graded series of ethanol, critical point dried and sputter coated with Au-Pd (80:20) using a Polaron E5000 sputter coater. Bacterial morphology was examined in a FEI Quanta 250 SEM using an SE detector at an accelerating voltage of 20 kV. At least 400 cells were analyzed for each sample from two independent experiments with xT Microscope control software (FEI).

Localization of peptides onto bacterial membrane

Localization and binding of the peptides onto bacteria were studied by using the rhodamine-labeled peptides⁸. Mid-log phase grown *E. coli* ATCC 25922 ($\sim 10^8$) were incubated with rhodamine-labeled peptides in PBS for 30 min at 37 °C with gentle shaking. The cells were washed with PBS three times and then adhered on poly-L-Lysine coated glass coverslips for 30 minutes. The immobilized cells were fixed with 2% paraformaldehyde in PBS for 10 to 15 min. After extensive washing with PBS, the coverslips were mounted in FluorSaveTM mounting media. The images of the cells were taken with a Carl Zeiss LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany) using a Plan-Apochromat 63x/1.40-numerical aperture oil differential interference contrast (DIC) objective lens with appropriate excitation and emission filters after exciting the fluorescent probe with a 561nm diode-pumped solid state (DPSS) laser line.

Tryptophan blue-shift assay and quenching of its emission by acrylamide

In order to look into the binding of peptides to bacterial membrane mimetic, PC/PG lipid vesicles and localization of their tryptophan residues onto these lipid vesicles, their emission spectra were recorded in PBS and in the presence of small unilamellar vesicles (SUVs) composed of PC/PG (3:1, w/w)⁸. The tryptophan was excited at 280 nm and the emission was scanned from 300 to 400 nm in a quartz cuvette with a PerkinElmer LS55 fluorescence spectrometer. Blue-shift of tryptophan fluorescence spectrum was measured from the shift of emission maximum of a peptide from higher wavelength in PBS to a shorter wavelength in the presence of PC/PG vesicles.

The Trp environment of the peptide in lipid bilayers can reflect the interactions of the peptides with lipid vesicles. To examine the possible localization of Trp residue of the peptides in presence of PC/PG, we performed tryptophan fluorescence quenching experiments in the presence of tryptophan emission quencher, acrylamide as described earlier⁸.

Transmission electron microscopy (TEM)

7-10 μ l Sample (0.1 mg/ml in water) was deposited on freshly glow discharged carbon-coated copper grid and allowed to adsorb for approximately 1 minute. Excess solution was blotted off using a filter paper. The grids were negatively stained with 1% aqueous uranyl acetate for 30 seconds and air dried. Grids were observed under a JEOL JEM 1400 transmission electron microscope at 80 kV after complete gun alignment and astigmatism correction. Images were acquired using a Gatan Orius Digital 2K \times 2K CCD camera. The images were analyzed using Digital Micrograph software (Gatan, Inc.). At least four grids for each peptide were prepared and analyzed.

ATR-FTIR

ATR-FTIR spectra were recorded on a PerkinElmer Spectrum two instrument. The samples were prepared in chloroform. A set of 10 measurements (2 runs/sec each) was run for each sample and the average of the peaks was plotted. The runs were carried out at 37 $^{\circ}$ C.

Circular dichroism (CD) studies

The circular dichroism (CD) spectra of the A β ₂₉₋₄₀ variants A β ₂₉₋₄₀-V1 and A β ₂₉₋₄₀-V2 were recorded on JASCO J-1500 spectrometer in 1% SDS (mimicking the bacterial membrane environment)⁹. The spectrometer was calibrated routinely with 10-camphorsulphonic acid. The samples were scanned at room temperature (\sim 25 $^{\circ}$ C) with the help of a capped quartz cuvette of 2 mm path length at a wavelength range of 250–200 nm. An average of 3 scans was taken for each sample with a scan speed of 50 nm/min and data interval of 1 nm.

Effects of peptides on binding of FITC-LPS to THP-1 cells

Fluorescein isothiocyanate-labeled lipopolysaccharide (FITC-LPS, 1 μ g/ml) was incubated with peptides (10 μ M) for 30 min at 37 $^{\circ}$ C, afterward 5×10^5 THP-1 cells were treated with FITC-LPS and Peptide mixture for 30 min at 37 $^{\circ}$ C with gentle shaking. After 30 min, the cells were washed with phosphate-buffered saline to remove the unbound FITC-LPS from the samples. Binding of FITC-LPS to THP-1 cells in the presence of peptides was monitored by measuring the mean fluorescence intensity (MFI) of 10,000 cells for each sample. The untreated cells served as autofluorescence (negative control) and cells treated with FITC-LPS only served as positive control (maximum fluorescence) in log FL1 height in a BD FACSCalibur flow cytometer.

Detection of cytokine levels in supernatant

Enzyme-linked immunosorbent assays (ELISAs) were performed to detect the levels of secreted TNF- α and IL-1 β in LPS-induced (100 ng/ml) THP-1 cells in the presence of peptides after 4 and 12 h of incubations respectively⁷. The supernatants, collected from LPS-treated and untreated cells were considered for the maximum and minimum production of cytokine levels respectively to calculate the percent inhibition by the peptides. The concentrations of TNF- α and IL-1 β in the samples were assessed using ELISA kits according to the manufacturer's protocol. The experiments were repeated thrice and the average values of the cytokine concentrations determined are included in results. Similar ELISAs were performed to estimate the amount of TNF- α and IL-6 secreted by BALB/c mice blood, which was collected from the orbital sinus 4 h after LPS injection (i.p.) by using ELISA kits for mice TNF- α and IL-6.

Anti-LPS Survival assay

To evaluate the *in vivo* efficacy of the A β ₂₉₋₄₀ variants A β ₂₉₋₄₀-V1 and A β ₂₉₋₄₀-V2, survival assay of BALB/c mice was performed against a lethal dose of LPS (12 mg/kg). All the mice were divided into different experimental groups each having five animals for LPS and peptide administration. Mice of group 1 were injected with saline only (negative control group); whereas mice of group 2 were administered intraperitoneally (i.p.) with a lethal dose of LPS (12 mg/kg) alone (positive control group). Mice of group 3 and 4 were treated with 2 different doses of the variant A β ₂₉₋₄₀-V1 at 5 mg/kg and 10 mg/kg respectively whereas mice groups of 5 and 6 were treated with the variant, A β ₂₉₋₄₀-V2 at 5 mg/kg and 10 mg/kg respectively after 15 min of LPS administration. Survival and status of the mice, treated with only saline, LPS alone and LPS and varying doses of variants together was monitored for 7 days.

References

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Figure S1

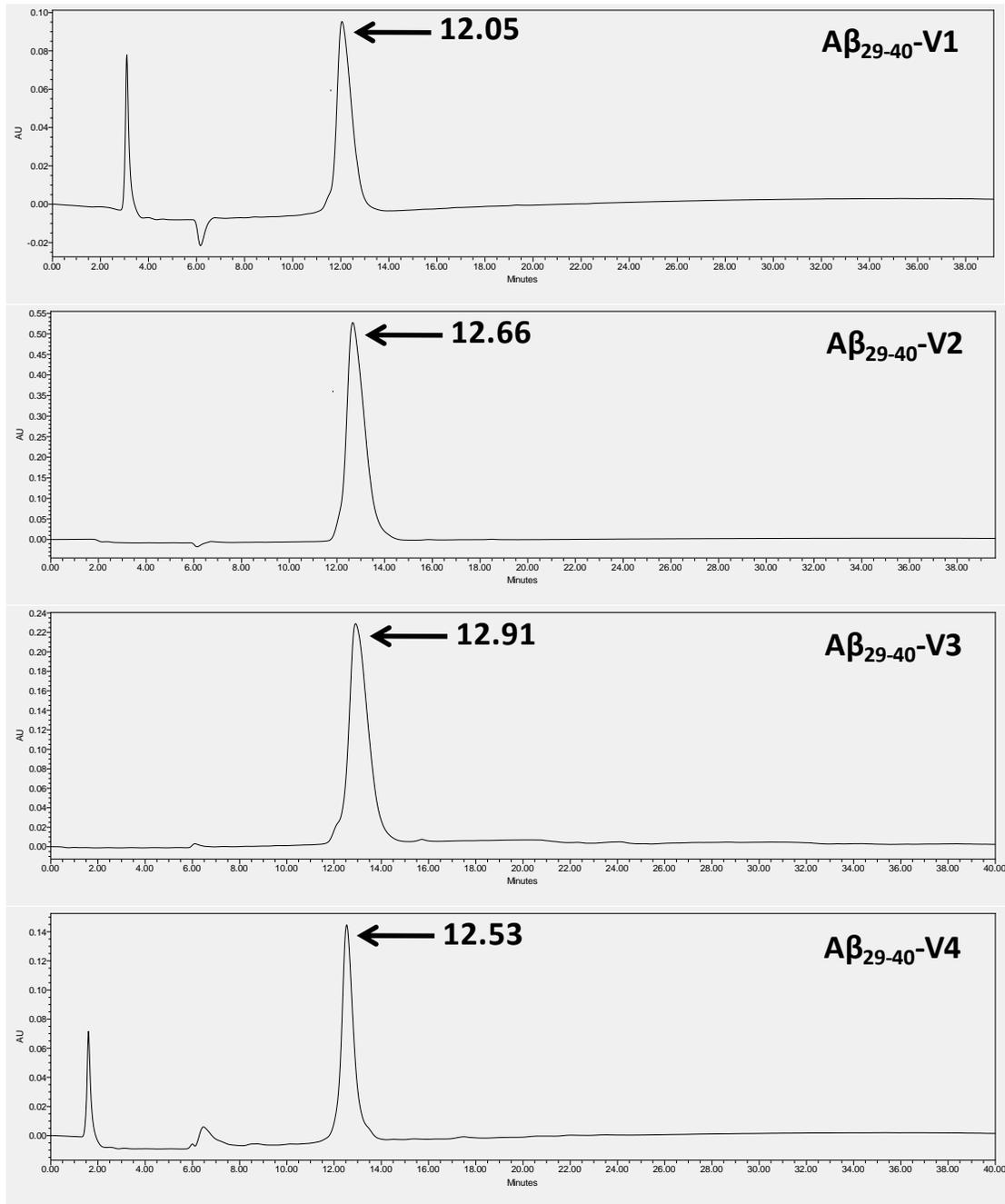


Figure S1. RP-HPLC traces of the Aβ₂₉₋₄₀ variants. All of the peptides were purified by RP-HPLC on a semi-preparative Waters C18 column using a linear gradient of 10-90% acetonitrile containing 0.1% TFA in 40 min with a flow rate of 2.0 ml/min. Arrow indicates the retention time of each of the peptide.

Figure S2

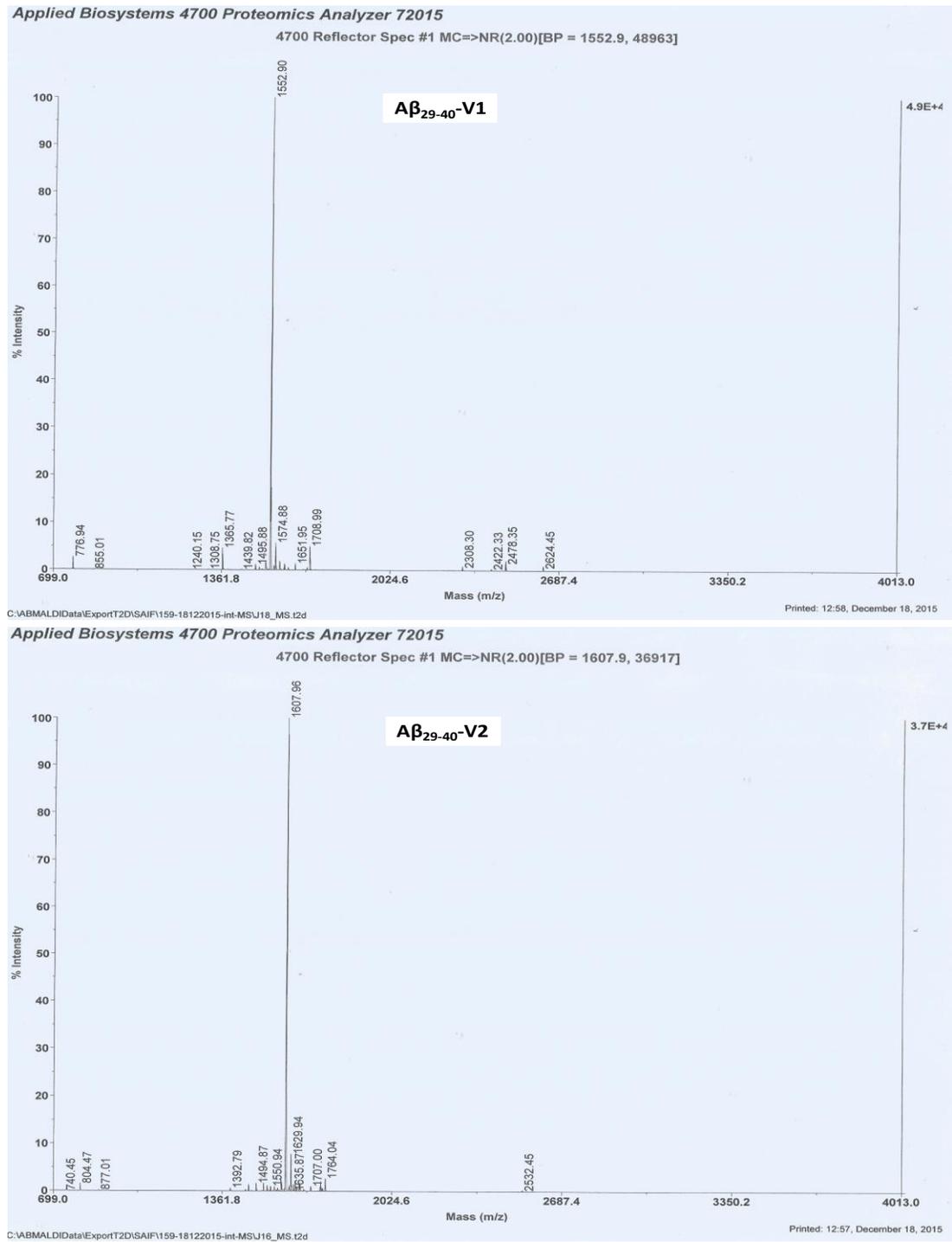


Figure S2. Mass spectra of A β ₂₉₋₄₀-V1 and A β ₂₉₋₄₀-V2. Mass of the variants was confirmed by MALDI-TOF.

Figure S3

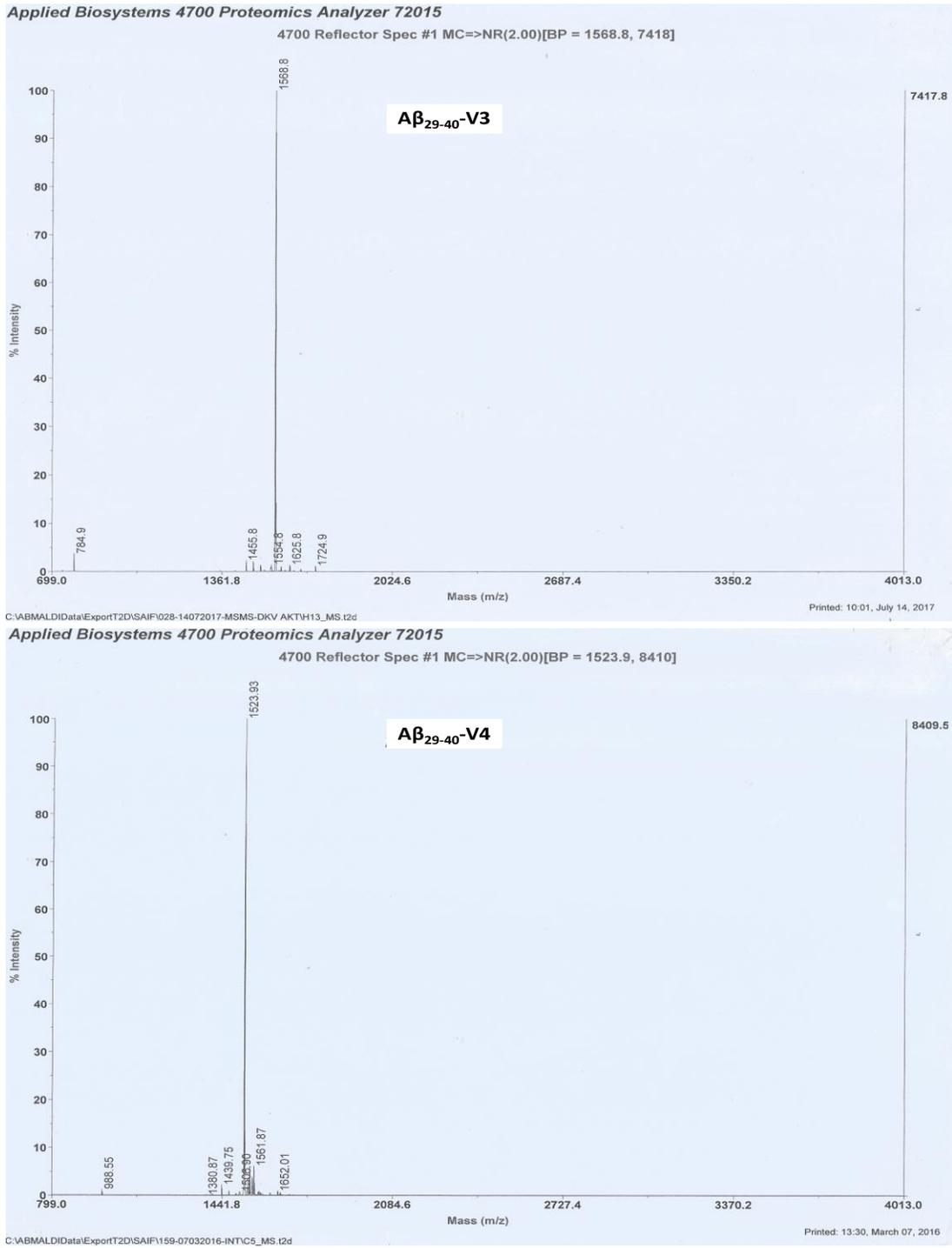


Figure S3. Mass spectra of A β ₂₉₋₄₀-V3 and A β ₂₉₋₄₀-V4.

Figure S4

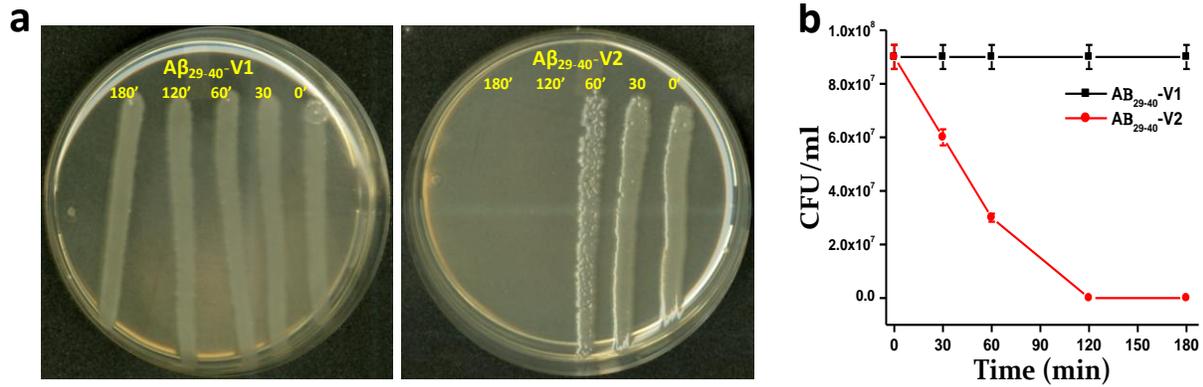


Figure S4. Killing kinetics of $A\beta_{29-40}\text{-V1}$ and $A\beta_{29-40}\text{-V2}$. **a**, Bacterial growth on MH-agar plates at different time points after treating of bacteria with peptides. **b**, Graphical presentation of bacterial CFU/ml in presence of the peptides at different time points.

Figure S5

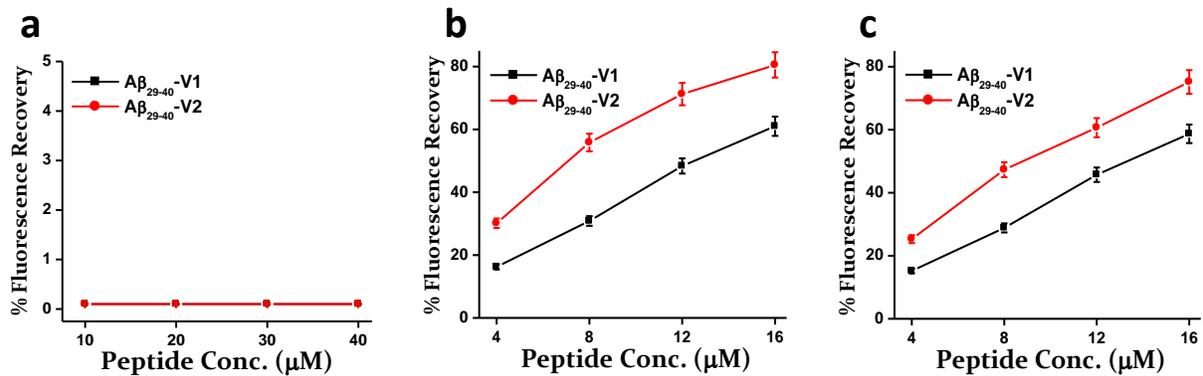


Figure S5. Peptide-induced transmembrane depolarization of hRBCs and bacteria. **a**, **b** and **c**, Plot of the percentage fluorescence recovery, which is a measure of peptide-induced transmembrane depolarization, against different peptide concentrations in hRBCs, *E. coli* and *S. aureus*, respectively.

Figure S6

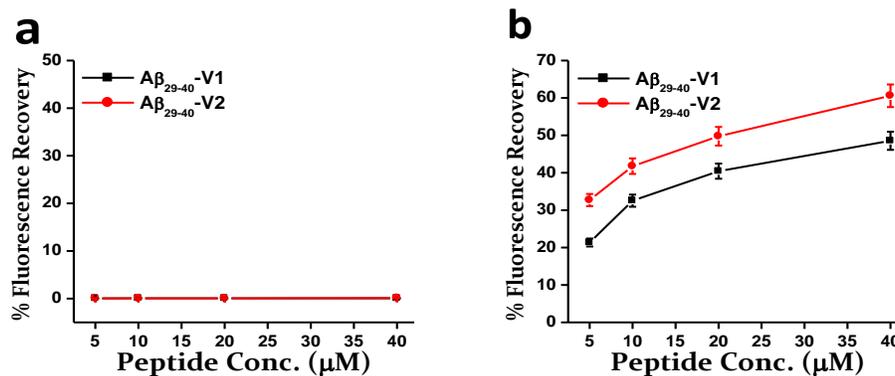


Figure S6. Dose-dependent peptide-induced transmembrane depolarization of lipid vesicles. **a**, Plot of the percentage fluorescence recovery, which is the measure of peptide-induced transmembrane depolarization, against the peptide concentration in mammalian membrane mimetic PC/Chol (8:1) lipid vesicles. **b**, Plot of the percentage fluorescence recovery in bacterial membrane mimetic PC/PG (3:1) lipid vesicles.

Figure S7

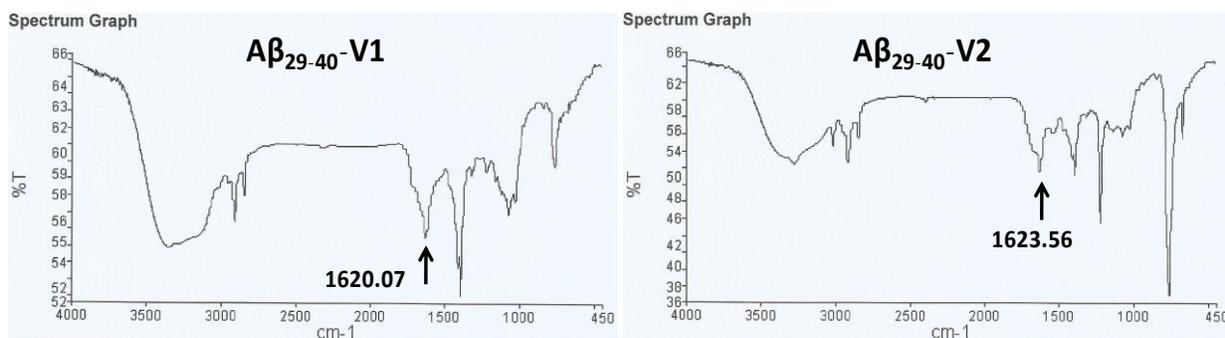


Figure S7. FTIR spectra of $\text{A}\beta_{29-40}\text{-V1}$ and $\text{A}\beta_{29-40}\text{-V2}$.

Figure S8

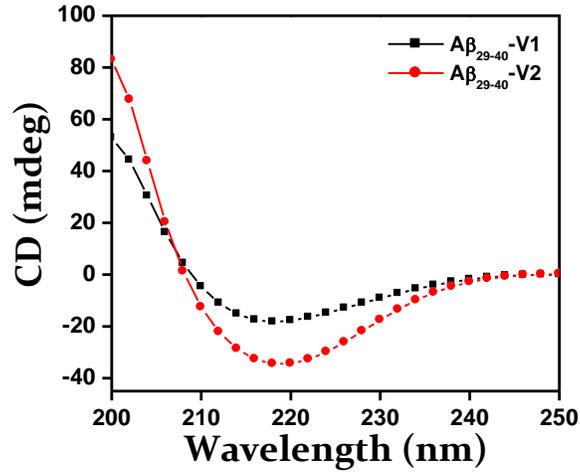


Figure S8. Determination of secondary structure of Aβ₂₉₋₄₀-V1 and Aβ₂₉₋₄₀-V2 in the presence of 1% sodium dodecyl sulfate (SDS). Negative peaks at ~ 218 nm indicate that these peptides adopt appreciable β-sheet structures.

Figure S9

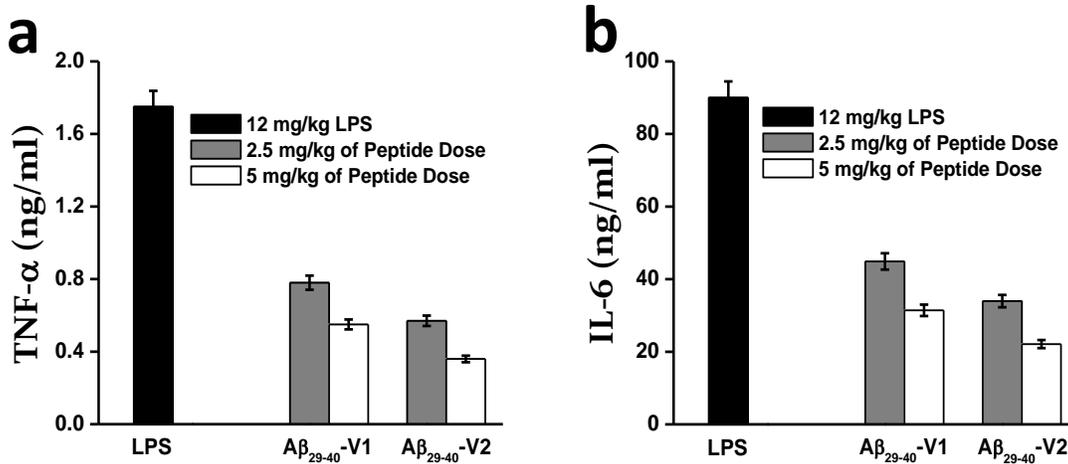


Figure S9. *In vivo* cytokine estimation of LPS treated mice. (a) and (b), Levels of TNF-α and IL-6 in serum of mice treated with LPS (12 mg/kg) in the absence and presence of Aβ₂₉₋₄₀-V1 and Aβ₂₉₋₄₀-V2 at different doses (2.5 mg/kg and 5 mg/kg), respectively.