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Mechanism of interaction of optimized *Limulus*-derived cyclic peptides with endotoxins.

Thermodynamical, biophysical, and microbiological analysis

Jörg Andrä*, Jörg Howe*, Patrick Garidel†, Manfred Rössle‡, Walter Richter§, José Leiva-León||, Ignacio Moriyon||, Rainer Bartels*, Thomas Gutschmann*, and Klaus Brandenburg*¹

*Forschungszentrum Borstel, Leibniz-Zentrum für Medizin und Biowissenschaften, Biophysik, Parkallee 10, 23845 Borstel, Germany

†Martin-Luther-Universität Halle-Wittenberg, Institut für Physikalische Chemie, Mühlpforte 1, 06108 Halle, Germany

‡European Molecular Biology Laboratory c/o DESY, Notkestr. 85, 22603 Hamburg, Germany

§Friedrich-Schiller-Universität Jena, Elektronenmikroskopisches Zentrum der Medizinischen Fakultät, Ziegmühlenweg 1, 07740 Jena, Germany

||Universidad de Navarra, Dept. de Microbiología, Irunlarrea 1, 31008 Pamplona, Spain

Abbreviations: LPS, lipopolysaccharide; FTIR Fourier-transform infrared spectroscopy; FRET fluorescence resonance energy transfer spectroscopy; MNC mononuclear cells; LALF *Limulus*-anti-lipopolysaccharide factor.

¹ **Corresponding author:** Tel: +49-(0)4537-188235; Fax: +49-(0)4537-188632

E-mail address: kbranden@fz-borstel.de

On the basis of formerly investigated peptides corresponding to the endotoxin-binding domain from the *Limulus* anti-lipopolysaccharide factor (LALF), a protein from *L. polyphemus*, we have designed and synthesized peptides of different lengths with the aim to get potential therapeutic agents against the septic shock syndrom. For an understanding of the mechanisms of action, we performed a profound physico-chemical and biophysical analysis of the interaction of rough mutant lipopolysaccharide (LPS) with these peptides by applying Fourier-transform infrared spectroscopy (FTIR), small-angle X-ray scattering (SAXS), calorimetric techniques (differential scanning calorimetry DSC and isothermal titration calorimetry ITC), and freeze-fracture transmission electron microscopy (FFTEM). Also, the action of the peptides on bacteria of different origin in microbial assays was investigated. Using FTIR and DSC, our data indicated a strong fluidization of the lipid A acyl chains due to peptide binding, with a decrease of the endothermic melting enthalpy change of the acyl chains down to a complete disappearance in the 1:0.5 to 1:2 [LPS]:[peptide] molar ratio range. Via ITC, it was deduced that the binding is a clearly exothermic process which runs into saturation also at a [LPS]:[peptide] = 1:0.5 to 1:2 molar ratio. The data obtained with SAXS indicated a drastic change of the aggregate structures of LPS into a multilamellar stack, which was visualized in electron micrographs as hundreds of lamellar layers. This can be directly correlated with the inhibition of the LPS-induced production of tumor-necrosis-factor- α in human mononuclear cells, but not with the action of the peptides on bacteria.

Keywords: Antimicrobial peptide, LPS neutralization, cytokine induction, *Limulus* test, SAXS, FTIR, DSC, FFTEM

Running title:: Biophysics of cyclic peptide-endotoxin interaction

INTRODUCTION

Cell wall-bound molecules of bacteria such as lipopolysaccharide (LPS) may be released into the environment by the attack of the immune system or simply due to cell division, and may there interact with serum or membrane proteins such as soluble or membrane-bound lipopolysaccharide-binding protein (LBP) and CD14 [1-3]. In a following step, this interaction may then promote to specific receptors such as TLR4/MD2, which eventually leads to cell activation [4,5]. At low and moderate concentrations of LPS the following inflammation reaction is beneficial for the host, but at higher LPS concentrations the cascade of molecular and cellular events may lead to the endotoxic shock syndrome with high mortality particularly in critical care units [6,7]. It is known that lipid A, the hydrophobic moiety anchoring LPS into the outer membrane of Gram-negative bacteria, constitutes the 'endotoxic principle' of LPS. Enterobacterial lipid A consists of a diglucosamine backbone phosphorylated at positions 1 and 4' to which mainly six acyl chains are linked at positions 2,3 and 2',3'. Furthermore, in all LPS chemotypes to lipid A two special monosaccharides, 2-keto-3-deoxyoctonate (Kdo) are linked, which are negatively charged due to their carboxylate groups. Thus, a necessary condition for LPS neutralization is the presence of positive charges, which can be achieved by the use of cationic peptides [8]. Previously, we have synthesized peptides based on the *Limulus*-anti-LPS-factor (LALF) from the horseshoe crab *Limulus polyphemus* [9]. These partial structures containing the LPS-binding domain or parts of it were found to have high endotoxin-binding and neutralizing activities, comparable to that of the parent recombinant rLALF protein (also called endotoxin-neutralizing protein ENP) [10], and to be non-toxic for erythrocytes or cultured human monocytes [11].

Based on these peptides, we have synthesized a number of cyclic peptides and have performed a detailed biophysical characterization of their interaction with mutant Ra LPS from *Salmonella minnesota* strain R60, which has well-defined chemical structure and

corresponds to the active unit in the heterogeneous mixtures of wild-type LPS [12]. In some cases also deep rough mutant Re LPS (from *S. minnesota* strain R595) as well as the 'endotoxic principle' lipid A were used. Information was obtained with regard to changes caused by peptide binding in LPS/lipid A aggregate structure, phase transition behaviour (temperature and enthalpy), mobilities of the acyl chains in the single phases, the binding epitopes, in particular the negative charges in lipid A, and the binding stoichiometry. Correlation of these results with data on the cytokine-inhibiting capacity of the peptides and their antimicrobial activity clearly established that the neutralizing activity of the peptides was linked to their affinity for LPS and to their ability to incorporate into target cell membranes. These data allow an understanding of the mechanism of the endotoxin:peptide interaction.

EXPERIMENTAL

Lipids

Lipopolysaccharides from rough mutant Re and Ra from *Salmonella minnesota* (strains R595 and R60, respectively) were extracted by the chloroform/petrol ether method [13] from bacteria grown at 37°C, purified, and lyophilized. Free lipid A was isolated by acetate buffer treatment of LPS R595. After isolation, the resulting lipid A was purified and converted into its triethylamine salt. Results of all the standard assays performed on the purified LPS and lipid A (analysis of the amount of glucosamine, total and organic phosphate, and the distribution of the fatty acid residues) were in good agreement with the chemical properties expected for LPS R595 and R60, whose molecular structure has already been solved [14].

Preparation of endotoxin aggregates

LPS or lipid A was solubilized in the appropriate buffer (lipid concentration 1 to 10 mM, depending on the applied technique), extensively vortexed, sonicated for 30 min in a water

bath, and subjected to several temperature cycles between 20 and 60 °C. Finally, the lipid suspension was incubated at 4 °C for at least 12 h before use.

Peptides

Peptides containing the described LPS-binding domain of LALF [15] termed cLALF9,12, 15,16, 18, and 20 (structures see Fig. 1) were synthesized with an amidated C-terminus by the solid-phase peptide synthesis technique on an automatic peptide synthesizer (model 433 A; Applied Biosystems) on the standard Fmoc-amide resin according to the fastmoc synthesis protocol of the manufacturer. The N-terminal Fmoc-group was removed from the peptide-resin and the peptide was deprotected and cleaved with 90 % trifluoroacetic acid (TFA), 5 % anisole, 2 % thioanisole, 3 % dithiothreitol for 3 h at room temperature. After cleavage the suspension was filtered and the soluble peptides were precipitated with ice-cold diethylether followed by centrifugation and extensive washing with ether. Peptides were purified by RP-HPLC using an Aqua-C18 column (Phenomenex). Elution was done by using a gradient of 0-70% acetonitrile in 0.1 % (TFA). Cyclation via cysteine residues was achieved by incubating the peptides in 10 % dimethylsulfoxid (DMSO) for 24 h at room temperature. The peptides were then again purified by reversed-phase HPLC to purities above 95%. Purity was determined by matrix-assisted laser-desorption-time-of-flight mass spectrometry (MALDI-TOF MS, Bruker). In some cases also peptide cLALF22, corresponding to the complete LPS-binding domain of LALF [16], was used. It differs from cLALF20 by the additional amino acids HY between the GC and the RI residues (third and fourth amino acid from the N-terminus [Fig. 1]).

Fourier-transform infrared spectroscopy (FTIR)

The infrared spectroscopic measurements were performed on an IFS-55 spectrometer (Bruker). Samples, dissolved in 20 mM Hepes buffer, pH 7.0, were placed in a CaF₂ cuvette

with a 12.5 μm teflon spacer. Temperature-scans were performed automatically between 10 and 70 $^{\circ}\text{C}$ with a heating-rate of 0.6 $^{\circ}\text{C}/\text{min}$. Every 3 $^{\circ}\text{C}$, 50 interferograms were accumulated, apodized, Fourier transformed, and converted to absorbance spectra.

Differential Scanning Calorimetry (DSC)

DSC measurements were performed with VP-DSC and MC-2 calorimeters (MicroCal, Inc., Northhampton, MA, USA) at a heating and cooling rate of 1 $^{\circ}\text{C}\cdot\text{min}^{-1}$. The DSC samples were prepared by dispersing a known amount (~ 3 mg/ml) in 10 mM PBS buffer at pH 7.4. The samples were hydrated in the liquid crystalline phase by vortexing. Prior to the measurements the DSC samples were stored for a defined time at 4 $^{\circ}\text{C}$ (see before). The measurements were performed in the temperature interval from 5 $^{\circ}\text{C}$ to 95 $^{\circ}\text{C}$. In the figures only the temperature range is shown where phase transitions were observed. Five consecutive heating and cooling scans checked the reproducibility of the DSC experiments of each sample [17]. The accuracy of the DSC experiments was ± 0.1 $^{\circ}\text{C}$ for the main phase transition temperatures and ± 1 $\text{kJ}\cdot\text{mol}^{-1}$ for the main phase transition enthalpy. The DSC data were analysed using the Origin software. The phase transition enthalpy was obtained by integrating the area under the heat capacity curve [18].

Isothermal Titration Calorimetry (ITC)

Microcalorimetric measurements of peptide binding to endotoxins were performed on a MCS isothermal titration calorimeter (Microcal Inc.) at 37 $^{\circ}\text{C}$ as recently described [19]. Endotoxin samples (0.05 to 0.15 mM) - prepared as described above - were dispensed into the microcalorimetric cell (volume 1.3 ml) and peptide solutions in the concentration range 0.5 to 5 mM were filled into the syringe compartment (volume 100 μl), each after thorough degassing of the suspensions. After temperature equilibration, the peptides were titrated in 3 μl portions every 5 min into the lipid-containing cell under constant stirring, and the measured

heat of interaction after each injection measured by the ITC instrument was plotted versus time. As addition of the peptides into pure buffer solution gave only negligible heat of dilution, the total heat signal from each experiment was determined as the area under the respective single peaks and plotted versus the [peptide]:[lipid] molar ratio. Since the instrument works in temperature equilibrium at a constant 'current feedback' corresponding to a power of approximately 74 μW , the occurrence of an exothermic reaction leads to a lowering of this current, whereas an endothermic reaction causes an increase. All titration curves were repeated at least twice.

X-ray diffraction

X-ray diffraction measurements were performed at the European Molecular Biology Laboratory (EMBL) outstation at the Hamburg synchrotron radiation facility HASYLAB using the double-focusing monochromator-mirror camera X33 [20]. Diffraction patterns in the range of the scattering vector $0.1 < s < 4.5 \text{ nm}^{-1}$ ($s = 2 \sin \theta / \lambda$, 2θ scattering angle and λ the wavelength = 0.15 nm) were recorded at 40 °C with exposure times of 1 min using an image plate detector with online readout (MAR345, MarResearch, Norderstedt/Germany). The s -axis was calibrated with Ag-Behenate which has a periodicity of 58.4 nm. The diffraction patterns were evaluated as described previously [21] assigning the spacing ratios of the main scattering maxima to defined three-dimensional structures. The lamellar and cubic structures are most relevant here. They are characterized by the following features:

(1) Lamellar: The reflections are grouped in equidistant ratios, i.e., 1, 1/2, 1/3, 1/4, etc. of the lamellar repeat distance d_l

(2) Cubic: The different space groups of these non-lamellar three-dimensional structures differ in the ratio of their spacings. The relation between reciprocal spacing $s_{hkl} = 1/d_{hkl}$ and lattice constant a is

$$s_{hkl} = [(h^2 + k^2 + l^2) / a]^1/2$$

(hkl = Miller indices of the corresponding set of plane).

Freeze-fracture transmission electron microscopy (FFTEM)

LPS and LPS:peptide samples were prepared as described above around 90 % water content (3-5 mg/ 30 μ l). For freeze-fracturing, the samples, copper sandwich profiles, and instruments for manipulation were incubated at room temperature or at 40 °C. A small amount of the sample was sandwiched between two copper profiles as used for the double-replica technique and frozen by plunging the sandwiches immediately into liquified propane cooled in liquid nitrogen. Fracturing and replication were performed at -150 °C in a BAF 400T freeze-fracture device (BAL-TEC, Balzers, Liechtenstein) equipped with electron guns and a film sheet thickness monitor. The replicas were placed on copper grids, cleaned with a chloroform-methanol mixture, and examined in an EM900 electron microscope (Zeiss, Oberkochen, Germany).

Fluorescence resonance energy transfer spectroscopy (FRET)

The fluorescence resonance energy transfer (FRET) technique was used as a probe dilution assay [22] to obtain information on the intercalation of the peptides in the absence and presence of LPS into liposomes made from phosphatidylserine (PS). For the FRET experiments, liposomes were double-labeled with NBD-PE and Rh-PE in chloroform [PS]:[NBD-PE]:[Rh-PE] at 100:1:1 molar ratios. The solvent was evaporated under a stream of nitrogen, the lipids resuspended in PBS, mixed thoroughly, and sonicated with a Branson sonicator for 1 min (1 ml solution). Subsequently, the preparation was temperature-cycled at least twice between 4 °C and 56 °C, each cycle followed by intense vortexing for a few min, and then stored at 4 °C for at least 12 h prior to measurement. A preparation of 900 μ l of the double-labeled liposomes (10^{-5} M) at 37 °C was excited at 470 nm (excitation wavelength of NBD-PE), and the intensities of the emission light of the donor NBD-PE (531 nm) and

acceptor Rh-PE (593 nm) were measured simultaneously on the fluorescence spectrometer SPEX F1T11 (SPEX Instr., Edison, NY). The peptides cLALF (final concentration 1 μ M) were added to liposomes after 50 s, and LPS after 100 s (cLALF:LPS 1:1 molar). Furthermore, coincubated (LPS + cLALF) samples were added after 50 s. Since FRET spectroscopy is used here as a probe dilution assay, intercalation of molecules causes an increase of the distance between donor and acceptor in the liposomes and, thus, leads to a reduced energy transfer. This again causes an increase of the donor and decrease of the acceptor intensities. For a qualitative analysis of experiments, the quotient of the intensities of the donor dye and the acceptor dye are plotted against time (denoted in the following as the FRET signal). The data shown are representative for three independent experiments.

Stimulation of human mononuclear cells by LPS

Mononuclear cells (MNC) were isolated from heparinized blood of healthy human donors as described previously [23]. The cells were resuspended in medium (RPMI 1640) and their number was equilibrated at 5×10^6 cells/ml. For stimulation, 200 μ l MNC (1×10^6 cells) were transferred into each well of a 96-well culture plate. LPS and peptide:LPS mixtures were incubated for 30 min at 37 °C, and added to the cultures at 20 μ l per well. The cultures were incubated for 4 h at 37 °C under 5 % CO₂. Supernatants were collected after centrifugation of the culture plates for 10 min at 400 x g and stored at -20 °C until determination of TNF α content. Immunological determination of TNF α was carried out in a Sandwich ELISA using a monoclonal antibody against TNF (clone 6b from Intex AG, Switzerland) and was described earlier in detail [23].

Assays for cytotoxicity

Cytotoxicity of the peptides was determined against HeLa cells in medium by a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as described previously [24].

Assay for hemolytic activity

Hemolytic activity of the peptides against human erythrocytes was determined as described previously [24]. Briefly, peptides were incubated with a freshly prepared erythrocyte suspension from human blood (5 % of normal hematocrit) in 10 mM phosphate buffered saline, pH 7.2, for 30 min at 37 °C. Released hemoglobin was determined photometrically at 412 nm.

Assays for antibacterial activities

The antibacterial activity of the peptides was determined by microdilution susceptibility assays performed i) in standard Mueller-Hinton broth or ii) in 20 mM Hepes buffer, pH 7.0 supplemented with 10% LB broth.

1) Assay in Mueller-Hinton broth. Susceptibility testing was performed following the recommendations of the Clinical Standards Institute (CLSI, formerly NCCLS) [Standards, 2000 [25]. Peptides (2 mg/ml in 4 mM HEPES, pH 7.2) were two-fold diluted in Mueller-Hinton Broth (ref. 275730, pH 7.3, Difco Laboratories, Le Pont de Claix, France) in 96-well microtitre plates (TPP, Trasadingen, Switzerland) to obtain concentrations from 512 to 0.25 µg/ml in a volume of 100 µl. Bacteria were grown on Mueller-Hinton agar plates (BioMérieux, Marcy l'Etoile, France) for 1 to 3 days depending on the growth rate of the bacteria, suspended in 2 ml of 0.9 % saline and adjusted to 10⁸ CFU/ml. The bacterial suspension was diluted 100-fold in Mueller-Hinton broth, and 0.1 ml of this new dilution (10⁵ CFU) was added to the peptide dilutions. The microtitre plates were incubated at 37 °C

for 24 h. The minimal inhibitory concentration (MIC) of each peptide against a given bacterial strain was regarded as the minimal concentration of the peptide which prevents the growth of that organism 24 h after inoculation. The bactericidal effect of the peptides was determined, after 24 h incubation at 37 °C, by plating 10 µl of the content of non-cloudy wells onto Mueller-Hinton agar plates. Plates were incubated at 37 °C for 1 to 5 days, and a viable count was performed. The minimal bactericidal concentration (MBC) of each peptide for a given bacterial strain was regarded as the minimal concentration of the peptide which kills 99.9 % of the colony-forming units (CFU) present in the final inoculum.

2) *Assays in Luria-Bertani (LB) medium / buffer.* Peptides (dissolved in 20 mM HEPES, pH 7.0) were two-fold diluted in the same buffer in 96-well microtitre plates (Greiner, Germany). Subsequently, a suspension of log-phase bacteria in LB was added (10 µl, containing 10⁴ CFU) to the peptide solution (90 µl). The plates were incubated overnight in a wet chamber at 37 °C under constant shaking and bacterial growth was monitored by measuring the absorbance at 620 nm in a microtiter plate reader (Rainbow, Tecan, Crailsheim, Germany). The MIC was defined as the lowest peptide concentration, at which no bacterial growth was measurable. Portions of each well (10 µl) were diluted with 90 µl HEPES buffer, plated out in duplicate on LB-agar plates, incubated overnight at 37 °C, and bacterial colonies were counted. The MBC was defined as the peptide concentration at which no colony growth was observed. Experiments were performed at least twice in duplicates.

RESULTS

Various physical techniques were applied to characterize the interaction of LPS Ra, in some cases also of LPS Re and lipid A as endotoxic principle, with the peptides.

Gel to liquid crystalline phase transition behavior

The influence of the peptides on the gel to liquid crystalline $\beta \leftrightarrow \alpha$ phase behaviour of the acyl chains of LPS Ra, as studied by DSC, showed for all peptides strong interaction with the LPS aggregates (Fig. 2). For all peptides, the endothermic melting enthalpy of the hydrocarbon chains of LPS decreases with increasing peptide concentration and vanished at equimolar ratios. The peptides cLALF15 and 20 acted strongest, for which the endotherm disappears already at a [LPS]:[peptide] 2:1 molar ratio. This became evident more clearly, when the enthalpy change ΔH of the acyl chain melting transition was plotted versus the [LPS Ra]:[peptide] molar ratio (Fig. 3). From these data it can be concluded that, beside the electrostatic attraction between the positive charges of the peptide and the negative charges of LPS, a hydrophobic interaction also takes place, leading to a complete abolishment of the chain melting. This chain melting-abolishing effect can be interpreted in two ways, as fluidization of the hydrocarbon chains already at low temperatures or as maintaining of the gel phase up to high temperatures. To differentiate between these possibilities, FTIR was applied by monitoring the peak position of the symmetric stretching vibration of the hydrocarbon chains ν_s (CH_2) versus temperature. This is plotted in Fig. 4 for the interaction of LPS Ra with cLALF9, 15, 18, and 20. As it is known that the peak position of ν_s (CH_2) lies around 2850 cm^{-1} in the gel and around 2852.5 to 2853 cm^{-1} in the liquid crystalline phases [26], it can be deduced from Fig. 4 that the action of the peptides led to a fluidization of the acyl chains in the gel phase, connected with a strong reduction of the phase transition temperature T_c . In particular at $37 \text{ }^\circ\text{C}$, a dramatic increase in the wavenumber values to more than 2853 cm^{-1} took place. Nevertheless, the change of the wavenumbers over a broad range $10\text{-}40 \text{ }^\circ\text{C}$ for the [LPS]:[peptide] mixtures at 1:0.5 and 1:1 molar ratio is a hint that in the DSC scans this transition cannot be observed enthalpically.

Binding experiments

Isothermal titration calorimetry is the method of choice to determine the enthalpy change caused by the peptide:LPS binding and its stoichiometry. For this, 0.05 mM LPS Ra dispersions were titrated with 2 mM peptide solutions. One original titration is shown in Fig. 5A for LPS Ra and cLALF20 showing an exothermic reaction (decrease of feedback power to compensate the temperature increase in the sample cell). A saturation is observed after the 10th injection (after approximately 2500 s). When plotting the integrated areas of the enthalpy curves (enthalpy change ΔH_c) versus [LPS]:[peptide] molar ratios (Fig. 5B), it can be deduced that (i), for all peptides the negative enthalpy changes ΔH_c corresponds to exothermic reactions; and that (ii), the titrations indicate a two-step process. Thus, for cLALF15 minima in the curve were observed at [LPS]:[peptide] = 0.3 and 1.6, respectively. Furthermore, binding saturation took place at highest molar ratio for cLALF9 ([LPS]:[peptide] = 2) and was lowest for cLALF20 ([LPS]:[peptide] < 1). The saturation values for the other peptides were intermediate between these two.

Ultrastructural analysis

It has been shown that non-lamellar aggregate structures for LPS (in particular the lipid A part of LPS) are essential for the expression of biological activity [27]. We thus applied synchrotron radiation small-angle X-ray diffraction to determine the aggregate structure of LPS in the presence of the peptides. For the latter, we used deep rough mutant LPS Re with a short core oligosaccharide rather than LPS Ra with the complete core oligosaccharide [28], since the spectra of the former are more readily resolvable and interpretable [29,30]. The diffraction patterns for pure LPS Re were indicative for the existence of a mixed unilamellar/cubic structure as described earlier [29] (data not shown). In the presence of the peptides cLALF18 and 20, the diffraction patterns in the temperature range 5 to 60 °C were characteristic for the existence of multilamellar structures, deduced from the occurrence of reflections at equidistant ratios (Fig. 6A). For the LPS:cLALF18 complex, a closer look to the

patterns at 40 °C (Fig. 6B) showed a main periodicity at 6.62 nm and three further reflections corresponding to the higher orders, and some smaller peaks. These were more readily expressed for the LPS:cLALF20 complex, showing a second lamellar periodicity at 5.32 nm and its second order at 2.64 nm (Fig. 6B, bottom).

Regarding the periodicities, it has been shown for LPS Re that these values lie in the range 5.90 to 6.40 in cases in which this LPS adopts a multilamellar structure (in the presence of Mg^{2+} or at low water content, [29]). Therefore, the addition of cLALF18 apparently leads to an increase in lamellar stacking periodicity, whereas that of cLALF20 does not, or is even decreased regarding the value of the smaller periodicity.

Also, the aggregate structures of free lipid A (as 'endotoxic principle') in the presence of cLALF18 and 20 were elucidated (data not shown). Similar to the case of LPS Re, the reflections at equidistant ratios indicated multilamellar structures. The value of the periodicities (6.49 nm), however, were very high regarding the fact that the values for pure lipid A are around 4.9 to 5.3 nm [31]. Thus, in this case a strong increase of the water layer between neighbouring bilayers due to peptide binding took place (swelling of the interlamellar repeat).

For the system LPS Re:cLALF18 (2.5:1 molar as in the X-ray diffraction experiments), also freeze-fracture electron microscopy was used. Micrographs are displayed in Fig. 7A-C. For pure LPS, vesicle-like structures are found in the size range 100 to 200 nm (Fig. 7A). The structures, however, often are not closed and formed something like 'open eggshells'. When the peptide was added to the LPS, the suspension became turbid with subsequent precipitation. The supernatant (Fig. 7B) as well as the precipitate (Fig. 7C) were investigated. It can be clearly seen that in the supernatant very large vesicle-like structures and multilamellar stacks are observed apparently due to the formation of fused 'eggshells', with diameters up to 500 nm. For the precipitate, the morphology completely changes with the occurrence of giant stacks. These stacks corresponded to the multilamellar structures found in

the X-ray experiments, with distances between neighboring stacks in the range 6.3 to 7.5 nm. Fig. 8C shows also that in cross-fractured areas stacks with more than 100 single bilayer lamellae can be found.

Intercalation into phospholipid liposomes

A possible intercalation of the peptides or peptide:LPS mixtures into phospholipid membranes were analysed by FRET. As shown in Fig. 8 for cLALF9 and cLALF18, the peptides alone (added at $t = 50$ s) were able to intercalate into PS liposomes (increase of FRET signal). When LPS was added subsequently at $t = 100$ s, there was an additional incorporation, in contrast to added LPS in the absence of the peptide, for which no incorporation was observed (data not shown, see [22]). This means that the peptides acted as a kind of transport molecule similar to that observed for LBP [22].

Finally, when peptides and LPS (peptide:LPS) were incubated before addition to the liposomes, the data indicate a reduced, but still existing membrane incorporation.

Similar results were obtained for all other peptides investigated (data not shown). In some experiments, instead of PS, phospholipids corresponding to the composition of the macrophage membrane ($PL_{M\phi}$) were used. The results were qualitatively the same as with pure PS liposomes, only the observed signal intensities were lower (data not shown).

Inhibition of LPS-induced stimulation of human mononuclear cells

We tested the ability of the peptides to inhibit cell stimulation by LPS Ra from *S. minnesota* strain R60. Cells were stimulated by LPS alone or preincubated with various amounts of peptide (Fig. 9). All peptides interacted with LPS as reflected by a reduction of cell activation. Peptide cLALF12 was able to completely block LPS-induced cell activation (1 ng/ml LPS) at

1 µg/ml. Polymyxin B, the gold standard for cationic LPS-neutralizing peptides, exhibited the same activity at a 10-fold lower concentration.

Cytotoxicity and hemolytic activity

The potential harmful effects of the peptides to human cells were estimated by assessing their cytotoxicity against HeLa cells and their potency to lyse human erythrocytes. At a concentration of 100 µg/ml none of the peptides were cytotoxic and the hemolytic activity was 15 % or below (Table 1).

Antibacterial activity

The antibacterial activity of the peptides was determined first against the LPS-mutant strains *S. minnesota* R595 and R60 (used for the preparation of the LPS with defined structures used in the above-described experiments) grown in LB, a low ionic strength medium that bolsters peptide antimicrobial activity. These experiments suggested that none of the new peptides outperformed the parent peptide cLALF22 (Table 1) but because of the low MICs obtained under these conditions and with these sensitive LPS mutant strains, we performed additional and more stringent experiments. To this end, we tested the antibacterial activity in Mueller-Hinton broth, which is rich in divalent cations, and using a smooth-type *Escherichia coli* strain and a clinical isolate of *Pseudomonas aeruginosa*, a bacterium with an outer membrane and LPS markedly stabilized by divalent cations. Under these more stringent conditions, the parent peptide cLALF22 outperformed again all the derivatives. This was particularly clear with *P. aeruginosa* highlighting the lack of an effective displacement of divalent cations from outer membrane and LPS targets by the other peptides (Table 1). It should be mentioned, however, that some of the variants exhibited activity against *S. aureus*. With respect to the most resistant bacterium, i.e. *P. aeruginosa*, cLALF9 was the peptide with highest activity beside cLALF22.

DISCUSSION

The development of suitable antimicrobial peptides for a therapy of septic shock and also the fight against local infections is still a challenge in human health care regarding the increase of infectious diseases worldwide and problems such as the emergence of resistances [32]. For these purposes, the development of drugs based on natural endotoxin-binding structures seems a promising approach. This approach comprises, for example, the use of lactoferrin-based [24] and of LALF (*Limulus*-anti-LPS factor)-based peptides [9]. For synthetic peptides based on the sequence of endotoxin-binding proteins like LALF, it was shown before, that some are in principle suitable for application in anti-sepsis research, in particular as they have a sufficiently long half-life in serum [33]. Previously, we have published biophysical data on the interaction of some synthetic cyclic peptides with LPS Re, and reported that certain physico-chemical parameters of the LPS Re:peptide interaction correlate with the ability to neutralize LPS [34]. On the basis of these findings, we have constructed several new cyclic peptides (cLALF 9, 12, 15, 16, 18 and 20). These peptides were designed on the basis of obtaining the hydrophobic cluster at the C-terminus and stepwise shortening the peptides from the N-terminus (Fig. 1). We have performed a comprehensive biophysical and biological study of their interaction with the well-characterized endotoxins LPS Re and LPS Ra from *S. minnesota* strains R595 and R60. Except for the ultrastructural studies in which LPS Re was used, the latter was studied because it has been shown that the endotoxically active unit in natural smooth form LPS corresponds to an LPS with complete core oligosaccharide [12].

Our results indicate a strong interaction of the peptides both with bacteria and with isolated LPS. The antibacterial activity of the peptides was clearly more marked on LPS deficient *Salmonella* mutants in low ionic strength broth than on wild-type clinical isolates in the divalent-cation rich Mueller-Hinton broth. In fact, under the more stringent conditions only the parent peptide exhibited activity on *P. aeruginosa* (Tab. 1). It has to be noted, however,

that this bacterium is notoriously refractory to bactericidal peptides under these conditions, and this highlights the activity of cLALF22. On the other hand, the peptides were not cytotoxic and only slightly hemolytic (Tab. 1). In a similar way, they were able to inhibit the LPS-induced cytokine production of human mononuclear cells (Fig. 9). Very interesting is the dependence of the antibacterial action on the presence of divalent cations (Mueller-Hinton broth). The latter are known to stabilize bacterial outer membranes as well as isolated LPS aggregates [34]. Therefore, the action of those peptides which essentially attack the LPS layer of the outer membrane is impeded, which explains the reduction of the MIC/MBC values (Tab. 1).

The binding of the peptides with LPS shows the following features, which may be relevant for an understanding of the binding to isolated LPS as well as to LPS as constituent of the outer bacterial membrane.

The binding led to a strong fluidization of the hydrocarbon chains of LPS (Fig. 4) with a disappearance of the phase transition enthalpy (Fig. 2,3) similar to the observations made for the well-known decapeptide polymyxin B (PMB) [19]. Concomitantly, a change of the aggregate structures of LPS and lipid A, being unilamellar or mixed unilamellar/inverted cubic in pure form, into multilamellar stacks (Figs. 6 and not shown) were observed, also like PMB. This process is strongly exothermic (Fig. 5A, B) due to the electrostatic attraction between the negative charges of LPS/lipid A and the positive charges of the peptides (in particular amino acids Arg=R and Lys=K, Fig. 1). The enthalpic values (maximum values more than 40 kJ/mol, see Fig. 5B) also compare well with those from the interaction of PMB and the nonapeptide PMBN with LPS [35].

The electron micrographs (Fig. 7) give a clear impression of the underlying morphology, with nearly spherical vesicle-like structures ('open eggshells', Fig. 7A) for pure LPS, and very large 'giant' liposomal structures in the supernatant (Fig. 7B), apparently due to fusions of the single 'eggshells'. In the precipitate, huge stacks were found (Fig. 7C) corresponding to

the multilamellae deduced from SAXS experiments. As already proposed in a preceding paper, the degree of multilamellarization may be a direct measure for the ability of a given peptide to inhibit the LPS-induced cytokine production in mononuclear cells [24]. According to theoretical considerations by Bouwstra et al. [36], from the shapes of the reflections at equidistant ratios statements about the number of lamellae and size distributions of particles should be possible. Unfortunately, this is apparently valid only for relatively simple situations for pure phospholipids. For LPS, in cases in which multilamellar aggregates are formed (at low water content and/or high Mg^{2+} concentrations [29,30]), we did not succeed to get these informations (unpublished data). Furthermore, due to the fact that for LPS:peptide mixtures we did not see spherical liposomes but rather relatively different forms in the supernatant and completely different morphologies in the precipitate, the data analysis of Bouwstra and co-workers is not applicable. Therefore, SAXS measurements alone are not sufficient for a complete analysis, and the number of lamellae must be estimated by a different technique, i.e., by analysing the FFTEM pictures. For cLALF18, Fig. 7C is indicative that stacks with very high numbers of lamellae are formed, and these can be enumerated (most of them are > 100). It is tempting to assume that this is relevant for the interpretation of biological data, since the interaction of LPS with binding-proteins such as CD14 and LBP is reduced for stacks with high number of lamellae as compared to the unilamellar system in the absence of the peptide. This will be a task for the future.

We have found here a significant increase of the aggregate sizes of LPS (Fig. 7c) concomitant with a multilamellarization (Fig. 6) in contrast to the findings of Rosenfeld et al [37], which report that peptides such as human LL-37 and magainin would lead to a dissociation of the LPS aggregates thus preventing it to react with LBP or cellular receptors. These authors, however, use more indirect techniques such as flow cytometry with FITC-labelled LPS which does not represent a direct technique as shown here with the FFTEM method and furthermore, they have used chemically ill-defined smooth-form LPS which usually consists of a mixture

of fractions differing in the length of the sugar chains and the acylation of the lipid A part [12].

It must be emphasized that except for uncharacterized wild type LPS (S-LPS) in older papers [38] no electron micrographs of chemically characterized endotoxins have ever been published. For lipid A, previously we have characterized the non-lamellar structures using SAXS and FFTEM [39]. A combination of SAXS and cryo-TEM was applied to analyse the interaction of granulysin-derived peptides with endotoxins, in particular with LPS Ra from *S. minnesota* [40]. The authors found for pure LPS Ra fibrillary structures with cylindrical forms with a diameter of the fibrilles of 12 nm, whereas in the presence of a granulysin-derived peptide regularly-stacked ribbons emerged, which would correspond to the multilamellar stacks of LPS Re as seen here.

It is important to note that the results of the combined EM and SAXS measurements presented here allow the unequivocal elucidation of the underlying aggregate structures, which should be of uttermost use in the interpretation of the inhibition activity of the peptides.

For an understanding of the interaction mechanism, the FRET data (Fig. 8), which exhibit an intercalation of the peptides into liposomal membranes, are important. This may be of relevance for their antibacterial activity (Tab. 1) as well for the inhibition of the LPS-induced cytokine expression (Fig. 9). For the former, because the bacterial membranes may thus be heavily disturbed in their function, and for the latter, since the uptake of the peptide into the membranes of mononuclear cells can be assumed to be of relevance for their ability to secrete cytokines. There is considerable evidence, that the cell activation by LPS starts via a membrane step, induced by binding proteins such as LBP and CD14 [2, 41-44]. The detailed mechanisms, however, are not completely resolved. It was proposed that LBP accelerates the binding of LPS to CD14, and/or that LBP leads to a disintegration, possibly monomerization, of LPS aggregates [2, 45], which would imply that the presence of monomers or small aggregates of endotoxin are necessary for endotoxic activity in accordance to findings of

Takayama and co-workers [46]. In contrast, Mueller et al [47, 48] showed that aggregates are the biologically active units of endotoxins. We have found that the binding of LBP to LPS leads to a multilamellarization of LPS which is rather indicative of an increase rather than a decrease of aggregate sizes as found here for peptide binding (unpublished results). Independently of this question, it can be stated that LPS incorporated into target cell membranes represents a strong disturbance of the membrane architecture due to the conical geometry (shape) of its lipid A moiety, which triggers cell activation by leading to a conformational change of membrane proteins responsible for cell signalling such as TLR4 [5,49], MaxiK [50], or a complete receptor cluster [51,52]. Since the peptides incorporate also into the membrane, they may be able to change the conical shape of LPS by inducing a lamellar geometry as it was seen in the X-ray diffraction experiments for LPS Re (Fig. 6) and lipid A (not shown).

CONCLUSION

The ability of the peptides to act antimicrobially, to inhibit the LPS-induced cytokine secretion without exhibiting cytotoxicity and hemolysis make them or derivatives thereof potential candidates in sepsis therapy. In the present paper the biophysical prerequisites for effective endotoxin neutralization were described: An exothermic reaction between LPS and peptides leading to a fluidization of the lipid A acyl chains with concomitant disappearance of the gel to liquid crystalline phase transition, a multilamellarization of the aggregate structure of LPS with a very high number of bilayer stacks, and an incorporation of the peptides into target phospholipids as model of immune cell membranes.

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Table 1: Antibacterial, cytotoxic, and hemolytic activity of cLALF peptides

	<i>Sal. min.</i> R595 ¹	<i>Sal. min.</i> R60 ¹	<i>E. coli</i> ATCC 25922 ²	<i>P. aeruginosa</i> ²	<i>S. aureus</i> ²	HeLa (% cytotox)	hRBC (% hemoglobin release)
CLALF9	4 (8)	8 (16)	64 (64)	256 (256)	128 (256)	0	9
CLALF12	4 (16)	8 (32)	64 (128)	>256 (>256)	64 (128)	0	11
CLALF15	4 (32)	8 (32)	32 (32)	>256 (>256)	64 (64)	0	14
CLALF16	4 (16)	16 (32)	64 (64)	>256 (>256)	128 (128)	0	15
CLALF18	4 (16)	8 (32)	256 (256)	>256 (>256)	256 (256)	0	11
CLALF20	8 (16)	16 (64)	64 (128)	>256 (>256)	>256 (>256)	0	6
CLALF22	2 (4)	8 (16)	32 (32)	32 (64)	n.d.	0	19

Antibacterial activity is given as MIC (MBC) in µg/ml and was determined by microdilution assays in (1) 10% LB / 90 % buffer, and in (2) Müller-Hinton broth (see Materials and Methods). Cytotoxicity against HeLa cells and lysis of human erythrocytes (hRBC) was determined for 100 µg/ml peptide and was calculated relative to an untreated control.

Figure legends

Fig. 1: Amino acid sequences of the synthesized cyclic LALF peptides. The cyclation is done between the two cysteine residues as indicated for cLALF20.

Fig. 2: DSC heating-scans of LPS R60 in the presence of different concentrations of peptides cLALF9 to 20. The specific excess heat capacity $C_{p \text{ diff}}$ of the calorimetric sample cell as compared to the reference cell is plotted versus temperature. The peak positions directed upwards are characteristic for endothermic chain melting processes.

Fig. 3: Calorimetrically determined enthalpy change ΔH_c versus [LPS]:[peptide] molar ratio from DSC scans of Fig. 2.

Fig. 4: Gel to liquid crystalline phase behaviour of the hydrocarbon chains in the lipid A moiety of LPS in the absence and presence of the cLALF9,15,18, and 20 in FTIR experiments. Plotted is the peak position of the symmetric stretching vibration of the methylene groups versus temperature. The gel phase corresponds to wavenumber values around 2850 cm^{-1} , the liquid crystalline phase to values around 2852.5 to 2853.0 cm^{-1} .

Fig. 5: Isothermal calorimetric titration of 0.05 mM LPS Ra with 2 mM cLALF peptides ($30 \times 3 \mu\text{l}$ every 5 min).

(A) Original titration of LPS with cLALF20 (raw data).

(B) Integrated peak areas of the single titrations according to (A) versus [peptide]:[LPS] molar ratios for cLALF9, 15,18, and 20.

Fig. 6: Synchrotron radiation small-angle X-ray diffraction patterns of [LPS]:[peptide] mixtures ([LPS]:[cLALF] = 2.5:1 molar. The scattering vector $s = 1/d = 2 \sin\theta / \lambda$ (d = spacing ratio, θ = scattering angle, λ = wavelength = 0.15 nm) is plotted versus the logarithm of the scattering intensity $\log I$.
(A) at different temperatures and (B) at 40 °C , both for cLALF18 and 20.

Fig. 7: Freeze-fracture electron micrographs of LPS from *Salmonella minnesota* Re alone (A) and in the presence of cLALF18 ([LPS]:[cLALF] = 3:1: (B) Supernatant, (C) Precipitation.

Fig 8: Intercalation of peptide/LPS samples and Hepes buffer control into target cell liposomes made from phosphatidylserine (PS) in fluorescence resonance energy transfer spectroscopic (FRET) measurements. Presented is the FRET signal I_D/I_A versus time. The samples were added subsequently at 50 and 100 s to the liposomes.

Fig. 9: Concentration-dependent inhibition of the biological activity of LPS Ra from *S. minnesota* strain R60 by synthetic peptides. Peptides were incubated with 1 ng/ml LPS at the indicated peptide concentrations and used to activate human mononuclear cells. As a marker of LPS-induced cell activation, the production of the cytokine TNF α was determined by ELISA. Activation of the cells by LPS alone represents 100%. Polymyxin B (PMB) served as a control. Data presented are the mean of two independent cell stimulations from two different preparations and each stimulation performed in duplicate.

CLALF

```
9   GC           RLKWK   KFWCG
12  GC           FRRLKWK  KFWCG
15  GC           FRRLKWKYKGKFWCG
16  GC           TFRRLKWKYKGKFWCG
18  GC   KPTFRRLKWKYKGKFWCG
20  GCRIKPTFRRLKWKYKGKFWCG
    +-----+
```

Fig. 1

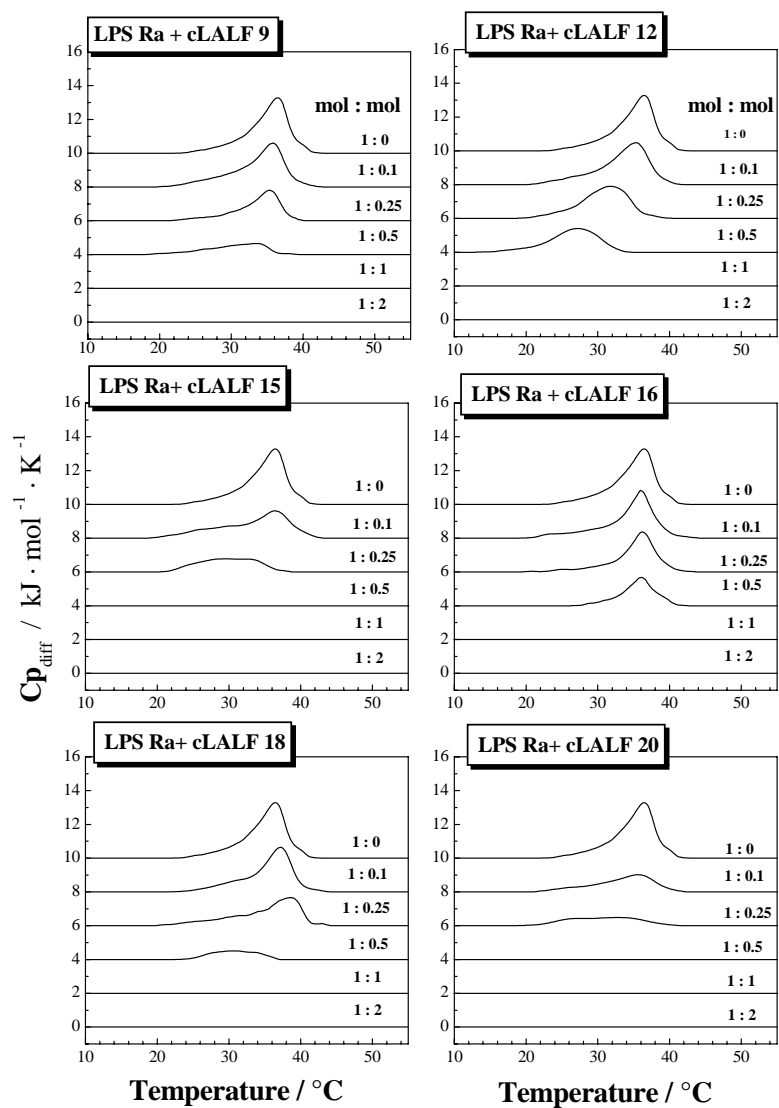


Fig. 2

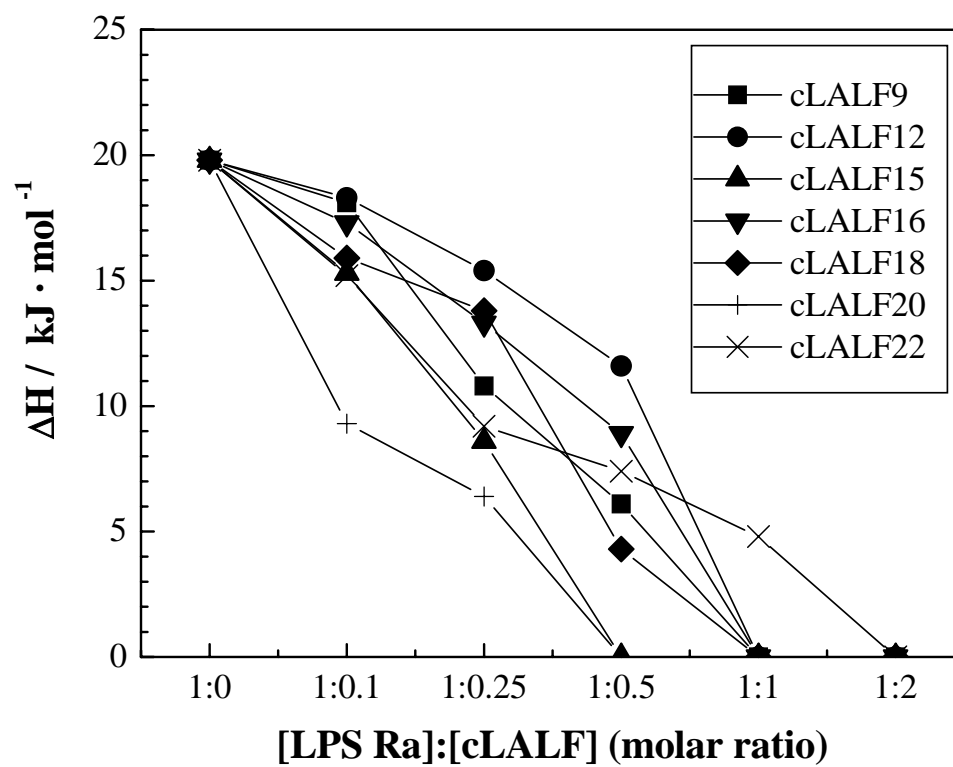
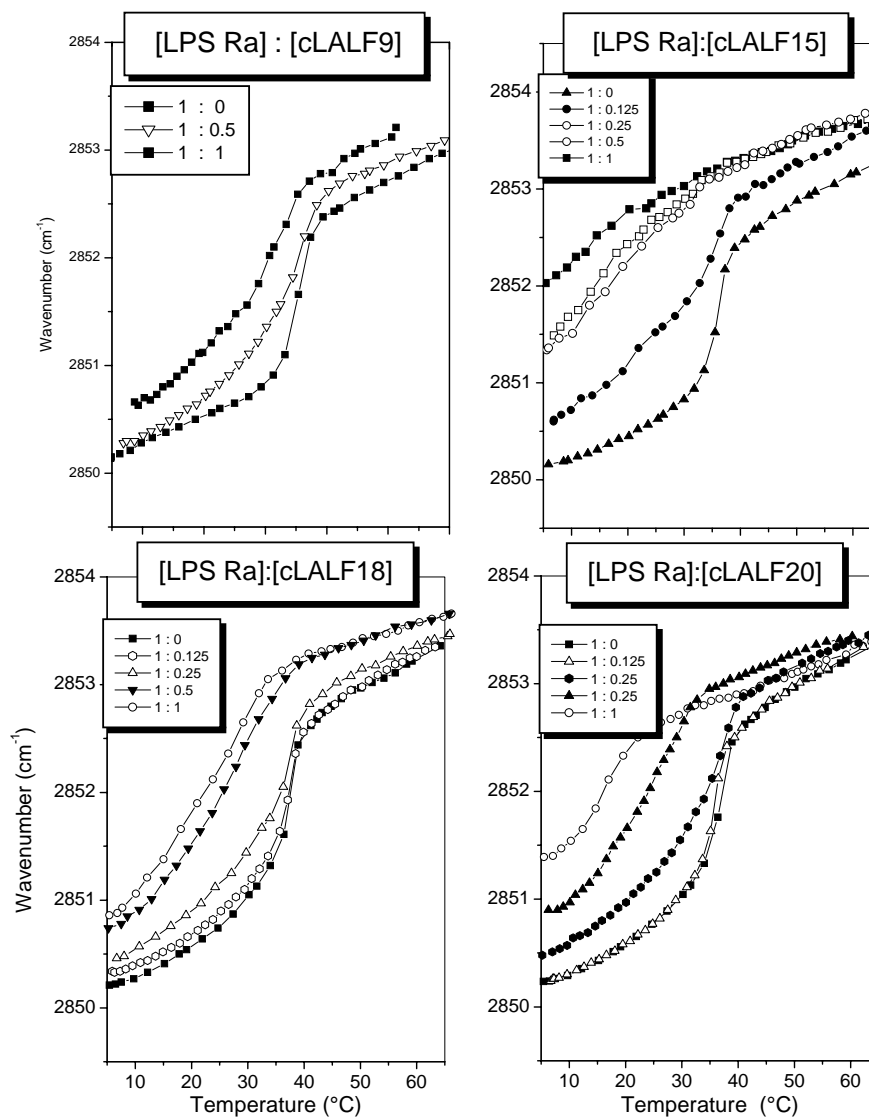


Fig. 3



7

Fig. 4

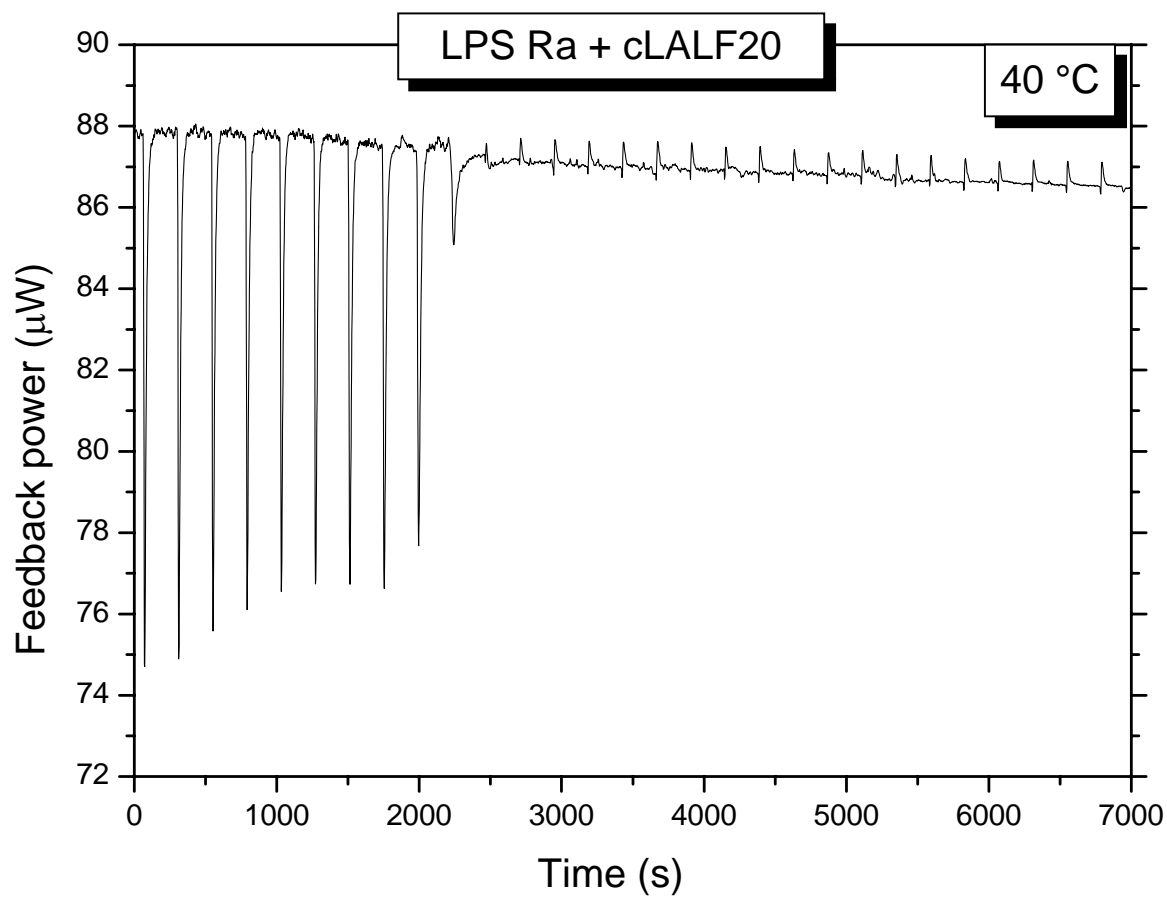


Fig. 5A

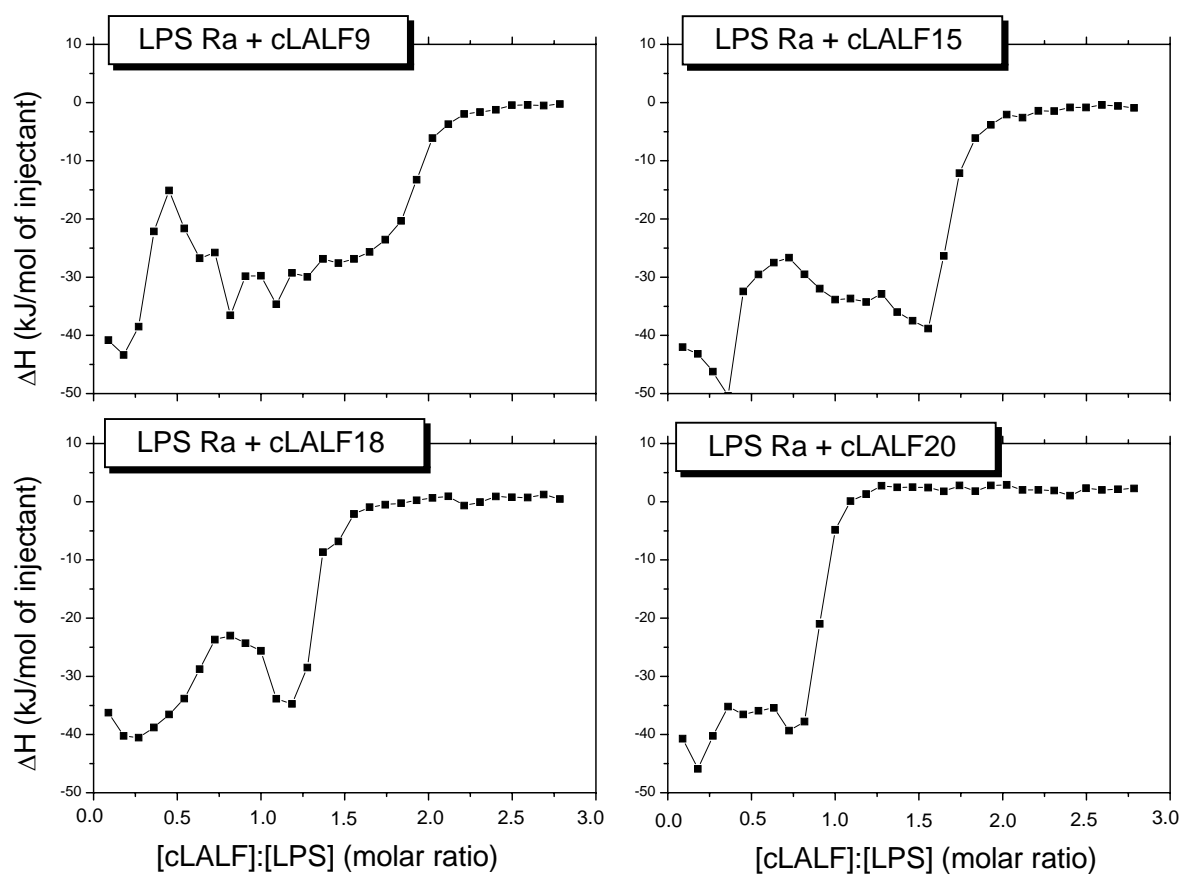


Fig. 5B

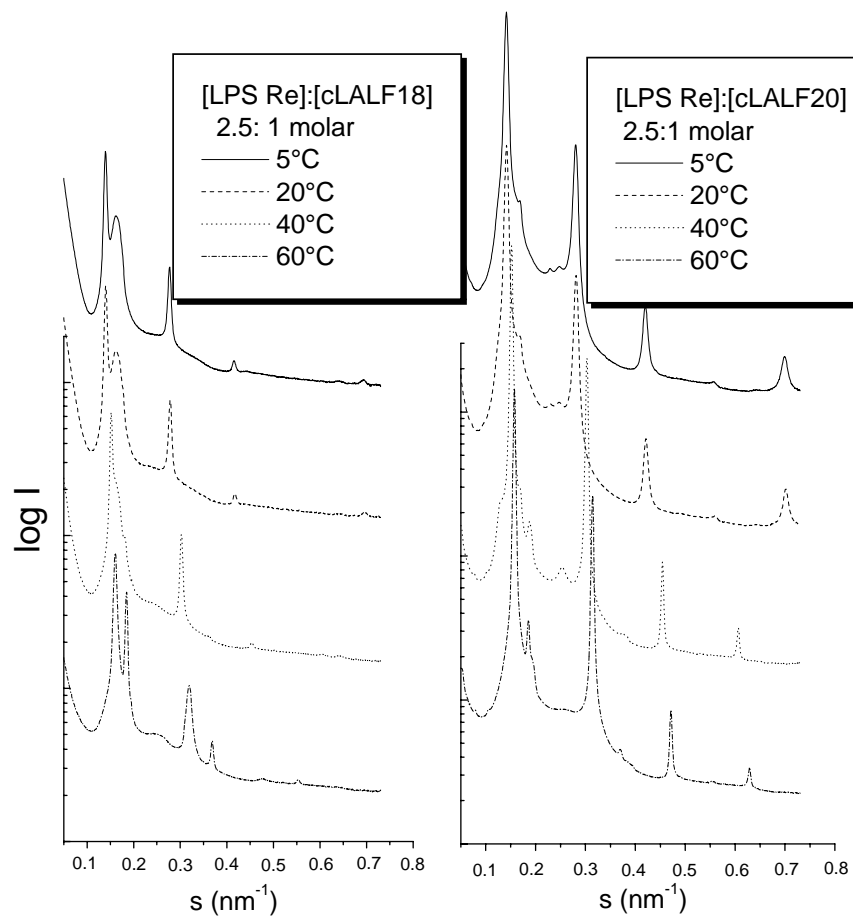


Fig. 6A

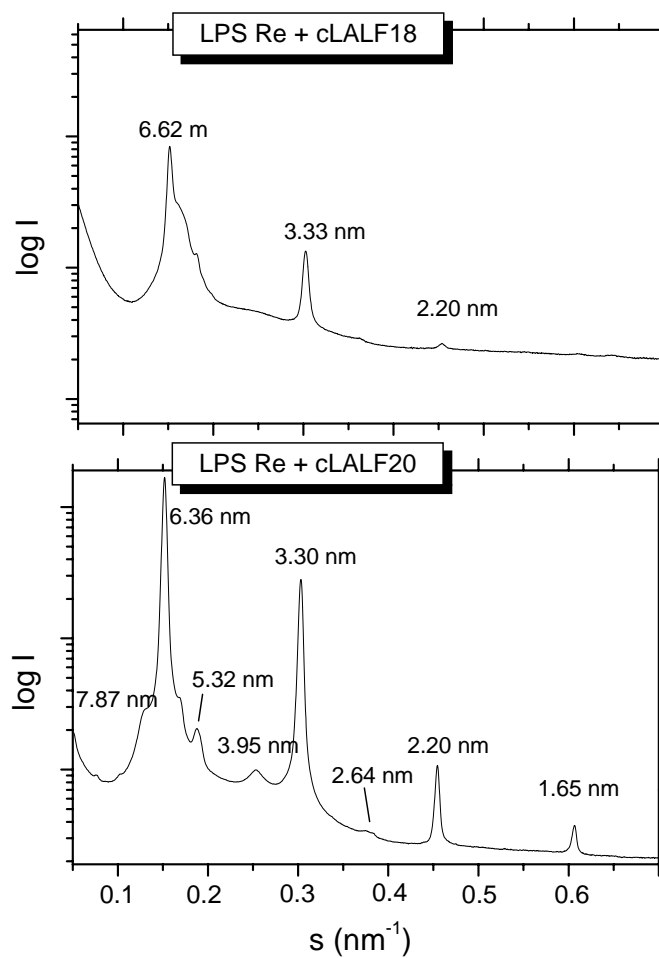


Fig. 6B

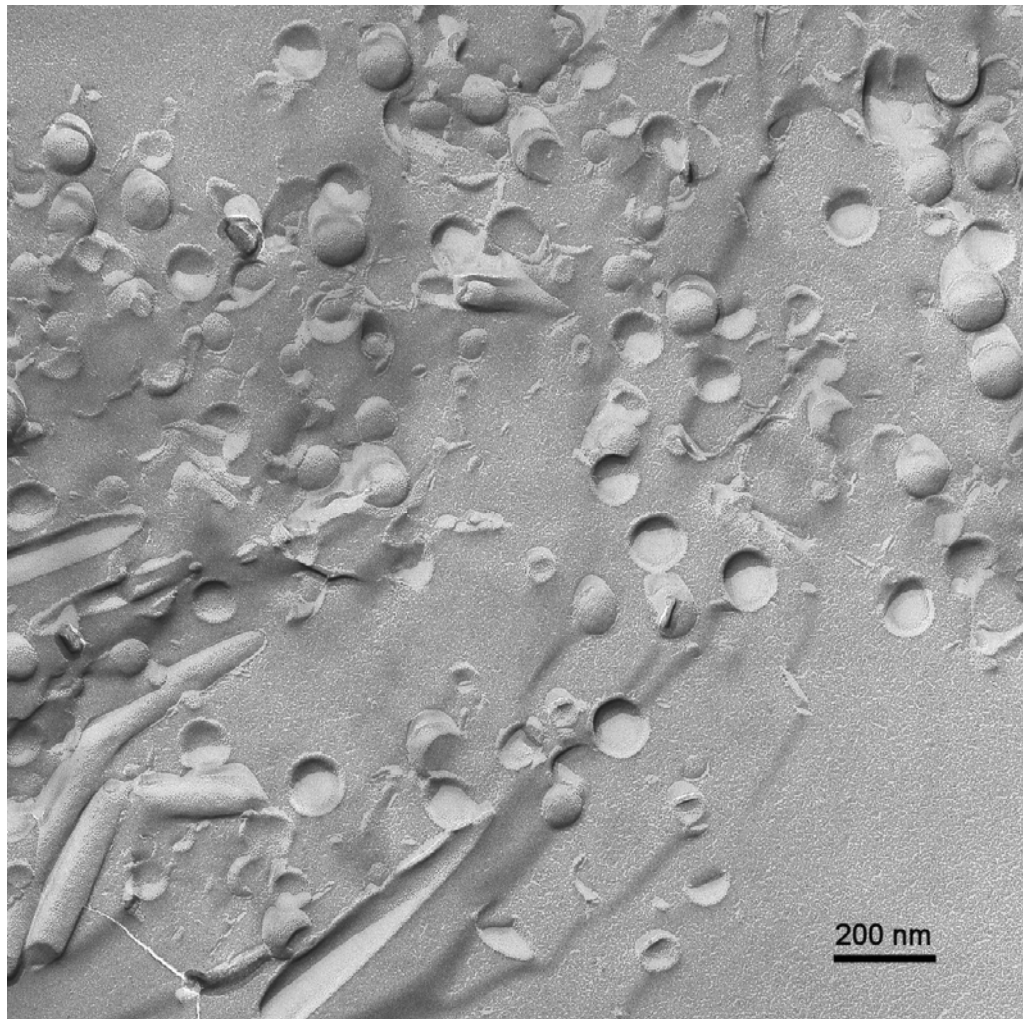


Fig. 7A

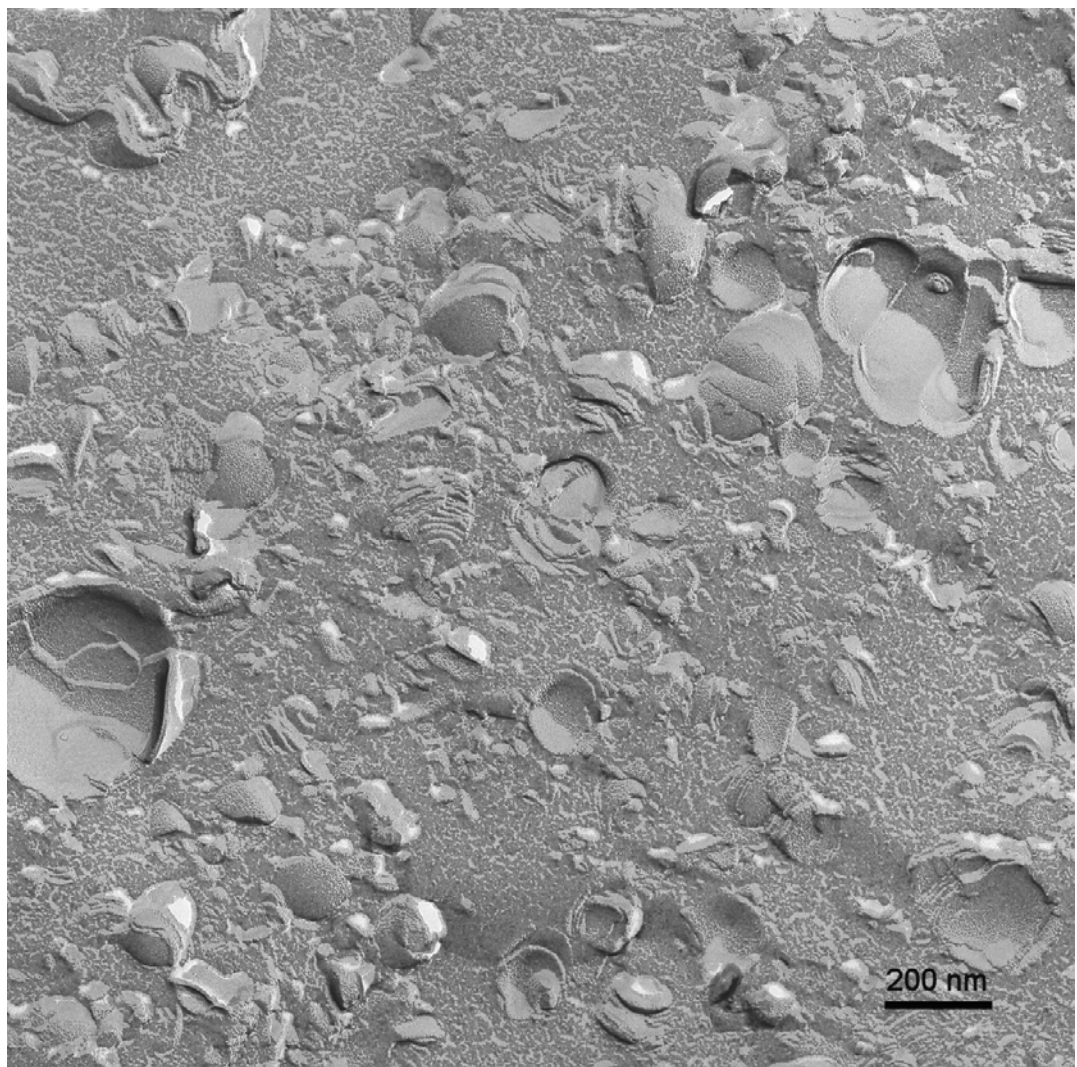


Fig. 7B

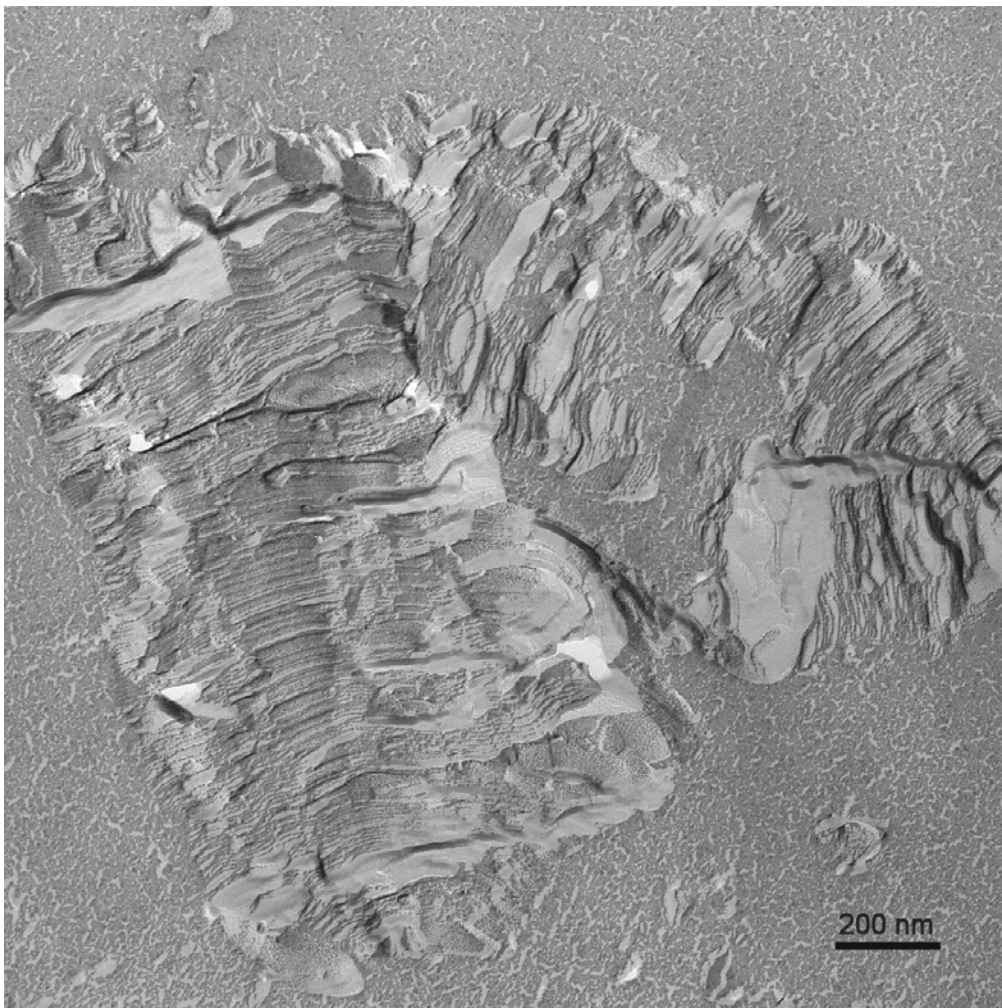


Fig. 7C

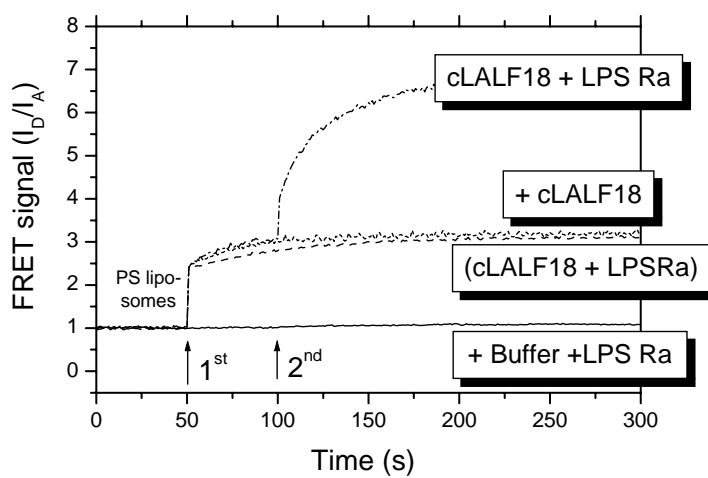
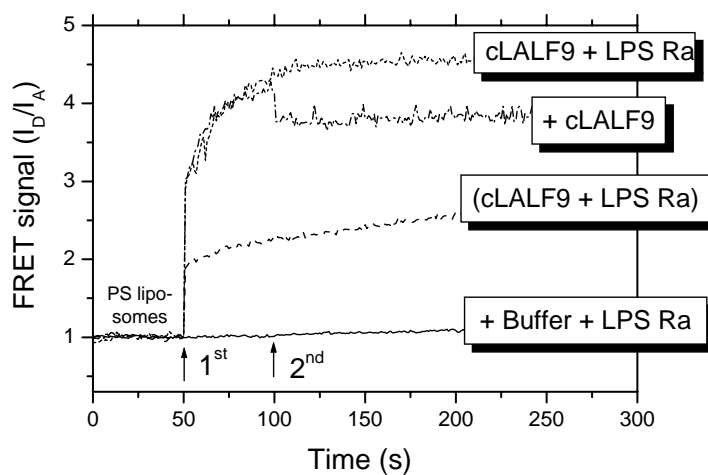


Fig.8

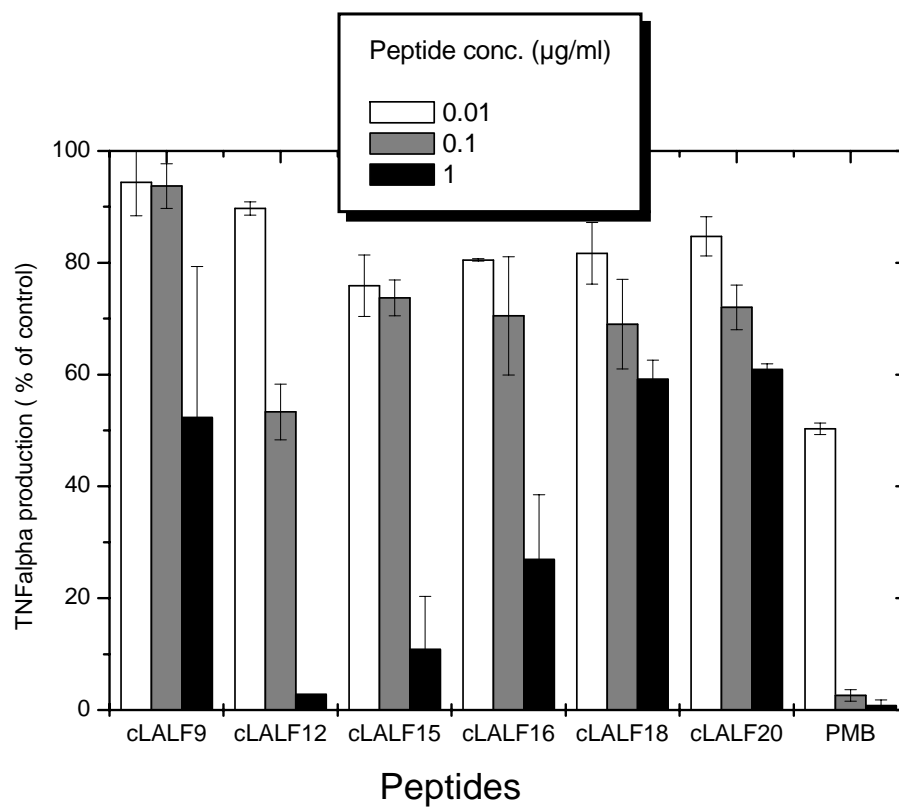


Fig.9