ITC XXV Endotoxin Binding Studies
(Lipid A, lipopolysaccharide, LPS)

Andra J., Lamata M., Martinez d. T., Bartels R., Koch M. H., and Brandenburg K. (2004) Cyclic antimicrobial peptides based on Limulus anti-lipopolysaccharide factor for neutralization of lipopolysaccharide. *Biochem Pharmacol* **68,** 1297-1307.

Abstract: Bacterial endotoxin (lipopolysaccharide, LPS) is responsible for the septic shock syndrome. As potential therapeutic agents cyclic cationic antimicrobial peptides of different length, based on the Limulus anti-lipopolysaccharide factor (LALF), were synthesized, and their interaction with LPS was characterized physico-chemically and related to results in biological assays. All peptides inhibited the LPS-induced cytokine production in human mononuclear cells and the Limulus amebocyte lysate in a concentration-dependent way, with the peptide comprising the complete LPS-binding loop of the LALF (cLALF22) being the most effective. The peptides were neither cytotoxic nor hemolytic, except a slight effect of cLALF22. The peptides were able to displace Ca(2+) cations from a LPS monolayer, with cLALF22 being again most effective in accordance with results from isothermal titration calorimetry, in which saturation of binding was observed at an equimolar [cLALF22]:[LPS] ratio, and at a ratio 2-2.5 for the other peptides. For cLALF22, zeta (xi) potential experiments exhibited a complete compensation of the negative charges of LPS, whereas for the other peptides a residual negative potential of -20 to -40mV was found. X-ray diffraction experiments showed that the mixed unilamellar/cubic inverted aggregate structure of the lipid A part of LPS was converted into a multilamellar one. The gel to liquid crystalline phase transition of the acyl chains of LPS was changed upon cLALF22 binding, leading to a clear fluidization, which was not observed or only to a lesser degree for the other peptides. The affinity of the peptides for LPS led to a reduced binding of lipopolysaccharide-binding protein (LBP) to target membranes and hence to an inhibition of cytokine induction in human mononuclear cells.

Andra J., Garidel P., Majerle A., Jerala R., Ridge R., Paus E., Novitsky T., Koch M. H., and Brandenburg K. (2004) Biophysical characterization of the interaction of Limulus polyphemus endotoxin neutralizing protein with lipopolysaccharide. *Eur J Biochem* **271,** 2037-2046.

Abstract: Endotoxin-neutralizing protein (ENP) of the horseshoe crab is one of the most potent neutralizers of endotoxins [bacterial lipopolysaccharide (LPS)]. Here, we report on the interaction of LPS with recombinant ENP using a variety of physical and biological techniques. In biological assays (Limulus amebocyte lysate and tumour necrosis factor-alpha induction in human mononuclear cells), ENP causes a strong reduction of the immunostimulatory ability of LPS in a dose-dependent manner. Concomitantly, the accessible negative surface charges of LPS and lipid A (zeta potential) are neutralized and even converted into positive values. The gel to liquid crystalline phase transitions of LPS and lipid A shift to higher temperatures indicative of a rigidification of the acyl chains, however, the only slight enhancement of the transition enthalpy indicates that the hydrophobic moiety is not strongly disturbed. The aggregate structure of lipid A is converted from a cubic into a multilamellar phase upon ENP binding, whereas the secondary structure of ENP does not change due to the interaction with LPS. ENP contains a hydrophobic binding site to which the dye 1-anilino-8-sulfonic acid binds at a K_d of 19 μM, which is displaced by LPS. Because lipopolysaccharide-binding protein (LBP) is not able to bind to LPS when ENP and LPS are preincubated, tight binding of ENP to LPS can be deduced with a K_d in the low nonomolar range. Importantly, ENP is able to incorporate by itself into target phospholipid liposomes, and is also able to mediate the intercalation of LPS into the liposomes thus acting as a transport protein in a manner similar to LBP. Thus, LPS-ENP complexes might enter target membranes of immunocompetent cells, but are not able to activate due to the ability of ENP to change LPS aggregates from an active into an inactive form.

Andra J., Lohner K., Blondelle S. E., Jerala R., Moriyon I., Koch M. H., Garidel P., and Brandenburg K. (2005) Enhancement of endotoxin neutralization by coupling of a C12-alkyl chain to a lactoferricin-derived peptide. *Biochem J* **385,** 135-143.

Abstract: Antibacterial peptide acylation, which mimics the structure of the natural lipopeptide polymyxin B, increases antimicrobial and endotoxin-neutralizing activities. The interaction of the lactoferricin-derived peptide LF11 and its N-terminally acylated analogue, lauryl-LF11, with different chemotypes of bacterial lipopolysaccharide (LPS Re, Ra and smooth S form) was investigated by biophysical means and was related to the peptides' biological activities. Both peptides exhibit high antibacterial activity against the

three strains of Salmonella enterica differing in the LPS chemotype. Lauryl-LF11 has one order of magnitude higher activity against Re-type, but activity against Ra- and S-type bacteria is comparable with that of LF11. The alkyl derivative peptide lauryl-LF11 shows a much stronger inhibition of the LPS-induced cytokine induction in human mononuclear cells than LF11. Although peptide-LPS interaction is essentially of electrostatic nature, the lauryl-modified peptide displays a strong hydrophobic component. Such a feature might then explain the fact that saturation of the peptide binding takes place at a much lower peptide/LPS ratio for LF11 than for lauryl-LF11, and that an overcompensation of the negative LPS backbone charges is observed for lauryl-LF11. The influence of LF11 on the gel-to-liquid-crystalline phase-transition of LPS is negligible for LPS Re, but clearly fluidizing for LPS Ra. In contrast, lauryl-LF11 causes a cholesterol-like effect in the two chemotypes, fluidizing in the gel and rigidifying of the hydrocarbon chains in the liquid-crystalline phase. Both peptides convert the mixed unilamellar/non-lamellar aggregate structure of lipid A, the 'endotoxic principle' of LPS, into a multilamellar one. These data contribute to the understanding of the mechanisms of the peptide-mediated neutralization of endotoxin and effect of lipid modification of peptides.

Andra J., Howe J., Garidel P., Rossle M., Richter W., Leiva-Leon J., Moriyon I., Bartels R., Gutsmann T. and Brandenburg K. (2007) Mechanism of interaction of optimized Limulus-derived cyclic peptides with endotoxins: thermodynamic, biophysical and microbiological analysis. *Biochem J* **406,** 297-307.
Abstract: On the basis of formerly investigated peptides corresponding to the endotoxin-binding domain from LALF [Limulus anti-LPS (lipopolysaccharide) factor], a protein from Limulus polyphemus, we have designed and synthesized peptides of different lengths with the aim of obtaining potential therapeutic agents against septic shock syndrome. For an understanding of the mechanisms of action, we performed a detailed physicochemical and biophysical analysis of the interaction of rough mutant LPS with these peptides by applying FTIR (Fourier-transform infrared) spectroscopy, SAXS (small-angle X-ray scattering), calorimetric techniques [DSC (differential scanning calorimetry) and ITC (isothermal titration calorimetry)] and FFTEM (freeze-fracture transmission electron microscopy). Also, the action of the peptides on bacteria of different origin in microbial assays was investigated. Using FTIR and DSC, our results indicated a strong fluidization of the lipid A acyl chains due to peptide binding, with a decrease in 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 becomes saturated at a 1:0.5 to 1:2 [LPS]:[peptide] molar ratio range. The results 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 tumour necrosis factor alpha in human mononuclear cells, but not with the action of the peptides on bacteria.

Bhunia A., Domadia P. N. and Bhattacharjya S. (2007) Structural and thermodynamic analyses of the interaction between melittin and lipopolysaccharide. *Biochim Biophys Acta* **1768,** 3282-3291.
Abstract: Lipopolysaccharide (LPS), the major constituent of the outer membrane of Gram-negative bacteria, is the very first site of interactions with the antimicrobial peptides. In this work, we have determined a solution conformation of melittin, a well-known membrane active amphiphilic peptide from honey bee venom, by transferred nuclear Overhauser effect (Tr-NOE) spectroscopy in its bound state with lipopolysaccharide. The LPS bound conformation of melittin is characterized by a helical structure restricted only to the C-terminus region (residues A15-R24) of the molecule. Saturation transfer difference (STD) NMR studies reveal that several C-terminal residues of melittin including Trp19 are in close proximity with LPS. Isothermal titration calorimetry (ITC) data demonstrates that melittin binding to LPS or lipid A is an endothermic process. The interaction between melittin and lipid A is further characterized by an equilibrium association constant (K(a)) of 2.85x10(6) M(-1) and a stoichiometry of 0.80, melittin/lipid A. The estimated free energy of binding (DeltaG(0)), -8.8 kcal mol(-1), obtained from ITC experiments correlates well with a partial helical structure of melittin in complex with LPS. Moreover, a synthetic peptide fragment, residues L13-Q26 or mel-C, derived from the C-terminus of melittin has been found to contain comparable outer membrane permeabilizing activity against Escherichia coli cells. Intrinsic tryptophan fluorescence experiments of melittin and mel-C demonstrate very similar emission maxima and quenching in presence of LPS micelles. The Red Edge Excitation Shift (REES) studies of tryptophan residue indicate that both peptides are located in very similar environment in complex with

LPS. Collectively, these results suggest that a helical conformation of melittin, at its C-terminus, could be an important element in recognition of LPS in the outer membrane.

Bhunia A., Chua G. L., Domadia P. N., Warshakoon H., Cromer J. R., David S. A. and Bhattacharjya S. (2008) Interactions of a designed peptide with lipopolysaccharide: Bound conformation and anti-endotoxic activity. *Biochem Biophys Res Commun* **369,** 853-857.
Abstract: Designed peptides that would selectively interact with lipopolysaccharide (LPS) or endotoxin and fold into specific conformations could serve as important scaffolds toward the development of antisepsis compounds. Here, we describe solution structure of a designed amphipathic peptide, H(2)N-YVKLWRMIKFIR-CONH(2) (YW12D) in complex with endotoxin as determined by transferred nuclear Overhauser effect spectroscopy. The conformation of the isolated peptide is highly flexible, but undergoes a dramatic structural stabilization in the presence of LPS. Structure calculations reveal that the peptide presents two amphipathic surfaces in its bound state to LPS whereby each surface is characterized by two positive charges and a number of aromatic and/or aliphatic residues. ITC data suggests that peptide interacts with two molecules of lipid A. In activity assays, YW12D exhibits neutralization of LPS toxicity with very little hemolysis of red blood cells. Structural and functional properties of YW12D would be applicable in designing low molecular weight non-toxic antisepsis molecules

Brandenburg K., David A., Howe J., Koch M. H., Andra J., and Garidel P. (2005) Temperature dependence of the binding of endotoxins to the polycationic peptides polymyxin B and its nonapeptide. *Biophys J* **88,** 1845-1858.
Abstract: The interaction between endotoxins-free lipid A and various lipopolysaccharide (LPS) chemotypes with different sugar chain lengths-and the polycationic peptides polymyxin B and polymyxin nonapeptide has been investigated by isothermal titration calorimetry between 20 and 50 degrees C. The results show a strong dependence of the titration curves on the phase state of the endotoxins. In the gel phase (<30 degrees C for LPS and <45 degrees C for lipid A), an endothermic reaction is observed, for which the driving force is an entropically driven endotoxin-polymyxin interaction, due to disruption of the ordered water structure and cation assembly in the lipid A backbone and adjacent molecules. In the liquid crystalline phase (>35 degrees C for LPS and >47 degrees C for lipid A) an exothermic reaction takes place, which is mainly due to the strong electrostatic interaction of the polymyxins with the negative charges of the endotoxins, i.e., the entropic change DeltaS is much lower than in the gel phase. For endotoxins with short sugar chains (lipid A, LPS Re, LPS Rc) the stoichiometry of the polymyxin binding corresponds to pure charge neutralization; for the compounds with longer sugar chains (LPS Ra, LPS S-form) this is no longer valid. This can be related to the lower susceptibility of the corresponding bacterial strains to antibiotics.

Brandenburg K., Jurgens G., Muller M., Fukuoka S., and Koch M. H. (2001) Biophysical characterization of lipopolysaccharide and lipid A inactivation by lactoferrin. *Biol Chem* **382,** 1215-1225.
Abstract: The interaction of bacterial endotoxins (LPS Re and lipid A, the 'endotoxic principle' of LPS) with the endogenous antibiotic lactoferrin (LF) was investigated using various physical techniques and biological assays. By applying Fourier-transform infrared (FTIR) spectroscopy, we find that LF binds to the phosphate group within the lipid A part and induces a rigidification of the acyl chains of LPS. The secondary structure of the protein - as monitored by the amide I band - is, however, not changed. Concomitant with the IR data, scanning calorimetric data indicate a sharpening of the acyl chain phase transition. From titration calorimetric and zeta potential data, saturation of LF binding to LPS was found to lie at a [LF]:[LPS] ratio of 1:3 to 1:5 M from the former and 1:10 M from the latter technique. X-ray scattering data indicate a change of the lipid A aggregate structure from inverted cubic to multilamellar, and with fluorescence (FRET) spectroscopy, LF is shown to intercalate by itself into phospholipid liposomes and may also block the lipopolysaccharide-binding protein (LBP)-induced intercalation of LPS. The LPS-induced cytokine production of human mononuclear cells exhibits a decrease due to LF binding, whereas the coagulation of amebocyte lysate in the Limulus test exhibited concentration-dependent changes. Based on these results, a model for the mechanisms of endotoxin inactivation by LF is proposed.

Brandenburg K., Koch M. H., and Seydel U. (1998) Biophysical characterisation of lysozyme binding to LPS Re and lipid A. *Eur J Biochem* **258,** 686-695.
Abstract: The binding of lysozyme to bacterial deep rough mutant lipopolysaccharide (LPS) Re and to its

lipid moiety lipid A, the 'endotoxic principle' of LPS, was investigated using biophysical techniques. The beta<-->alpha gel to liquid crystalline phase transition, the nature of the functional groups of the endotoxins, the secondary structure of lysozyme, and competition with polymyxin B were studied by Fourier-transform infrared spectroscopy (FTIR); the supramolecular aggregate structure of the endotoxins was determined with synchrotron radiation X-ray diffraction and the binding stoichiometry with microcalorimetry. The results were compared with those found with zwitterionic and negatively charged phospholipids. It can clearly be shown that lysozyme binds electrostatically to charged groups of the endotoxin molecules with the consequence of acyl-chain rigidification and an initiation of a transition from inverted cubic to multilamellar structures. The binding stoichiometry of endotoxin and lysozyme is a 3:1 molar ratio for both LPS Re and lipid A, indicating a dominant binding of lysozyme to the lipid A-phosphates. This could be confirmed by the analysis of a phosphate vibration and by the use of a dephospho LPS. Parallel to lysozyme binding to endotoxin, a conformational change of the secondary structure in the protein from mainly alpha helix to more unordered structures takes place, while the residual beta-sheet substructure does not exhibit a clear concentration dependence. Binding is found to be specific for the endotoxins since, for the zwitterionic phosphatidylcholine, no binding is observed and, for the negatively charged phosphatidylglycerol, only very weak binding is found. The results are discussed in the context of the ability of lysozyme to reduce endotoxicity.

Chen X., Howe J., Andra J., Rossle M., Richter W., da Silva A. P., Krensky A. M., Clayberger C. and Brandenburg K. (2007) Biophysical analysis of the interaction of granulysin-derived peptides with enterobacterial endotoxins. *Biochim Biophys Acta* **1768,** 2421-2431.
Abstract: To combat infections by Gram-negative bacteria, it is not only necessary to kill the bacteria but also to neutralize pathogenicity factors such as endotoxin (lipopolysaccharide, LPS). The development of antimicrobial peptides based on mammalian endotoxin-binding proteins is a promising tool in the fight against bacterial infections, and septic shock syndrome. Here, synthetic peptides derived from granulysin (Gra-pep) were investigated in microbiological and biophysical assays to understand their interaction with LPS. We analyzed the influence of the binding of Gra-pep on (1) the acyl chain melting of the hydrophobic moiety of LPS, lipid A, by Fourier-transform spectroscopy, (2) the aggregate structure of LPS by small-angle X-ray scattering and cryo-transmission electron microscopy, and 3) the enthalpy change by isothermal titration calorimetry. In addition, the influence of Gra-pep on the incorporation of LPS and LPS-LBP (lipopolysaccharide-binding protein) complexes into negatively charged liposomes was monitored. Our findings demonstrate a characteristic change in the aggregate structure of LPS into multilamellar stacks in the presence of Gra-pep, but little or no change of acyl chain fluidity. Neutralization of LPS by Gra-pep is not due to a scavenging effect in solution, but rather proceeds after incorporation into target membranes, suggesting a requisite membrane-bound step.

Fernandez M. M., Bhattacharya S., De Marzi M. C., Brown P. H., Kerzic M., Schuck P., Mariuzza R. A. and Malchiodi E. L. (2007) Superantigen natural affinity maturation revealed by the crystal structure of staphylococcal enterotoxin G and its binding to T-cell receptor Vbeta8.2. *Proteins* **68,** 389-402.
Abstract: The illnesses associated with bacterial superantigens (SAgs) such as food poisoning and toxic shock syndrome, as well as the emerging threat of purpura fulminans and community-associated methicillin-resistant S. aureus producer of SAgs, emphasize the importance of a better characterization of SAg binding to their natural ligands, which would allow the development of drugs or biological reagents able to neutralize their action. SAgs are toxins that bind major histocompatibility complex class II molecules (MHC-II) and T-cell receptors (TCR), in a nonconventional manner, inducing T-cell activation that leads to production of cytokines such as tumor necrosis factor and interleukin-2, which may result in acute toxic shock. Previously, we cloned and expressed a new natural variant of staphylococcal enterotoxin G (SEG) and evaluated its ability to stimulate in vivo murine T-cell subpopulations. We found an early, strong, and widespread stimulation of mouse Vbeta8.2 T-cells when compared with other SAgs member of the SEB subfamily. In search for the reason of the strong mitogenic potency, we determined the SEG crystal structure by X-ray crystallography to 2.2 A resolution and analyzed SEG binding to mVbeta8.2 and MHC-II. Calorimetry and SPR analysis showed that SEG has an affinity for mVbeta8.2 40 to 100-fold higher than that reported for other members of SEB subfamily, and the highest reported for a wild type SAg-TCR couple. We also found that mutations introduced in mVbeta8.2 to produce a high affinity mutant for other members of the SEB subfamily do not greatly affect binding to SEG. Crystallographic analysis and docking into mVbeta8.2 in complex with SEB, SEC3, and SPEA showed that the deletions and

substitution of key amino acids remodeled the putative surface of the mVbeta8.2 binding site without affecting the binding to MHC-II. This results in a SAg with improved binding to its natural ligands, which may confer a possible evolutionary advantage for bacterial strains expressing SEG.

Howe J., Andra J., Conde R., Iriarte M., Garidel P., Koch M. H., Gutsmann T., Moriyon I. and Brandenburg K. (2007) Thermodynamic analysis of the lipopolysaccharide-dependent resistance of gram-negative bacteria against polymyxin B. *Biophys J* **92,** 2796-2805.
Abstract: Cationic antimicrobial cationic peptides (CAMP) have been found in recent years to play a decisive role in hosts' defense against microbial infection. They have also been investigated as a new therapeutic tool, necessary in particular due to the increasing resistance of microbiological populations to antibiotics. The structural basis of the activity of CAMPs has only partly been elucidated and may comprise quite different mechanism at the site of the bacterial cell membranes or in their cytoplasm. Polymyxin B (PMB) is a CAMP which is effective in particular against Gram-negative bacteria and has been well studied with the aim to understand its interaction with the outer membrane or isolated membrane components such as lipopolysaccharide (LPS) and to define the mechanism by which the peptides kill bacteria or neutralize LPS. Since PMB resistance of bacteria is a long-known phenomenon and is attributed to structural changes in the LPS moiety of the respective bacteria, we have performed a thermodynamic and biophysical analysis to get insights into the mechanisms of various LPS/PMB interactions in comparison to LPS from sensitive strains. In isothermal titration calorimetric (ITC) experiments considerable differences of PMB binding to sensitive and resistant LPS were found. For sensitive LPS the endothermic enthalpy change in the gel phase of the hydrocarbon chains converts into an exothermic reaction in the liquid crystalline phase. In contrast, for resistant LPS the binding enthalpy change remains endothermic in both phases. As infrared data show, these differences can be explained by steric changes in the headgroup region of the respective LPS

Howe J., Hammer M., Alexander C., Rossle M., Fournier K., Mach J. P., Waelli T., Gorczynski R. M., Ulmer A. J., Zahringer U., Rietschel E. T. and Brandenburg K. (2007) Biophysical characterization of the interaction of endotoxins with hemoglobins. *Med. Chem* **3,** 13-20.
Abstract: Bacterial endotoxin (lipopolysaccharide, LPS) is the major component of the outer leaflet of the outer membrane in gram-negative bacteria. During severe infections, bacteria may reach the blood circuit of humans, and endotoxins may be released from the bacteria due to cell division or cell death. In particular enterobacterial forms of LPS represent extremely strong activator molecules of the human immune system causing a rapid induction of cytokine production in monocytes and macrophages. Various mammalian blood proteins have been documented to display LPS binding activities mediating normally decreasing effects in the biological activity of LPS. In more recent studies, the essential systemic oxygen transportation protein hemoglobin (Hb) has been shown to amplify LPS-induced cytokine production on immune cells. The mechanism responsible for this effect is poorly understood. Here, we characterize the interaction of hemoglobin with LPS by using biophysical methods. The data presented, revealing the changes of the type and size of supramolecular aggregates of LPS in the presence of Hb, allow a better understanding of the hemoglobin-induced increase in bioactivity of LPS.

Jurgens G., Muller M., Koch M. H., and Brandenburg K. (2001) Interaction of hemoglobin with enterobacterial lipopolysaccharide and lipid A. Physicochemical characterization and biological activity. *Eur J Biochem* **268,** 4233-4242.
Abstract: The interaction of hemoglobin (Hb) with endotoxins [i.e. with enterobacterial deep rough mutant lipopolysaccharide (LPS) Re and the "endotoxic principle" of LPS, lipid A] was investigated using a variety of physical techniques and with two biological assays, tumor necrosis factor (TNF)-alpha induction in human mononuclear cells and the Limulus amebocyte lysate (LAL) assay. Fourier-transform IR-spectroscopic experiments indicate nonelectrostatic binding to the hydrophobic moiety with a slight rigidification of the lipid A acyl chains, and an increase in the inclination of the lipid A backbone with respect to the membrane surface from 35 degrees to more than 40 degrees due to Hb binding, but no change of the predominantly alpha-helical secondary structures of Hb due to LPS binding. From isothermal titration calorimetry, the molar [Hb] : [endotoxin] binding ratio lies between 1 : 3 and 1 : 5 molar. Synchrotron radiation X-ray diffraction measurements indicate a reorientation of the lipid A aggregates from one cubic structure to another, the final structure belonging to space group Q224. The LPS-induced TNF-alpha production of mononuclear cells is enhanced by Hb, whereas in the LAL assay an LPS

concentration-dependent increase or decrease was observed. Although a detailed mechanism of action cannot be given, the enhancement of LPS bioactivity can be understood in the light of the previously presented conformational concept; Hb induces an increase in the conical shape of the lipid A moiety of LPS, higher cross-section of the hydrophobic than the hydrophilic part, and of the inclination angle of the diglucosamine backbone with respect to the direction of the acyl chains.

Jurgens G., Muller M., Garidel P., Koch M. H., Nakakubo H., Blume A., and Brandenburg K. (2002) Investigation into the interaction of recombinant human serum albumin with Re-lipopolysaccharide and lipid A. *J Endotoxin Res* **8,** 115-126.
Abstract: The interaction of bacterial endotoxins, deep rough mutant lipopolysaccharide LPS Re and the 'endotoxic principle' lipid A, with recombinant human serum albumin (rHSA) was investigated with a variety of physical techniques and biological assays. With Fourier-transform infrared spectroscopy and differential scanning calorimetry, the influence of albumin on the acyl chain melting behavior of the endotoxins was measured. Also, the effect on the functional groups of the endotoxins, in particular with respect to their orientation, was studied, including competition experiments with polymyxin B. Furthermore, the influence of endotoxin binding to rHSA on the protein's secondary structure was investigated. The results indicate a non-electrostatic binding with no change of the backbone orientation of LPS and only a slight change of the secondary structure of rHSA. Correspondingly, the amount of charge neutralization of the endotoxins due to rHSA measured by the electrophoretic mobility exhibited only a slight reduction of the surface potential. From these measurements and isothermal titration calorimetry, the lipid:protein binding stoichiometry was estimated to [LPS]:[rHSA], 10:1 molar. The determination of the aggregate structure of the endotoxins by X-ray small-angle scattering exhibited a complex change of a cubic into a non-lamellar structure. No influence of rHSA on endotoxin intercalation into phospholipid liposomes induced by lipopolysaccharide-binding protein could be detected by fluorescence resonance energy transfer. Finally, the LPS-induced cytokine production of human mononuclear cells was only slightly increased at high molar rHSA excess, while the coagulation of amebocyte lysate in the Limulus test yielded a complex change due to rHSA binding of LPS.

Kono M., Okumura Y., Tanaka M., Nguyen D., Dhanasekaran P., Lund-Katz S., Phillips M. C. and Saito H. (2008) Conformational flexibility of the N-terminal domain of apolipoprotein a-I bound to spherical lipid particles. *Biochemistry* **47,** 11340-11347.
Abstract: Lipid binding of human apolipoprotein A-I (apoA-I) occurs initially through the C-terminal alpha-helices followed by conformational reorganization of the N-terminal helix bundle. This led us to hypothesize that apoA-I has multiple lipid-bound conformations, in which the N-terminal helix bundle adopts either open or closed conformations anchored by the C-terminal domain. To investigate such possible conformations of apoA-I at the surface of a spherical lipid particle, site-specific labeling of the N- and C-terminal helices in apoA-I by N-(1-pyrene)maleimide was employed after substitution of a Cys residue for Val-53 or Phe-229. Neither mutagenesis nor the pyrene labeling caused discernible changes in the lipid-free structure and lipid interaction of apoA-I. Taking advantage of a significant increase in fluorescence when a pyrene-labeled helix is in contact with the lipid surface, we monitored the behaviors of the N- and C-terminal helices upon binding of apoA-I to egg PC small unilamellar vesicles. Comparison of the binding isotherms for pyrene-labeled apoA-I as well as a C-terminal helical peptide suggests that an increase in surface concentration of apoA-I causes dissociation of the N-terminal helix from the surface leaving the C-terminal helix attached. Consistent with this, isothermal titration calorimetry measurements showed that the enthalpy of apoA-I binding to the lipid surface under near saturated conditions is much less exothermic than that for binding at a low surface concentration, indicating the N-terminal helix bundle is out of contact with lipid at high apoA-I surface concentrations. Interestingly, the presence of cholesterol significantly induces the open conformation of the helix bundle. These results provide insight into the multiple lipid-bound conformations that the N-terminal helix bundle of apoA-I can adopt on a lipid or lipoprotein particle, depending upon the availability of space on the surface and the surface composition

Nguyen T. B., Suresh Kumar E. V., Sil D., Wood S. J., Miller K. A., Warshakoon H. J., Datta A. and David S. A. (2008) Controlling Plasma Protein Binding: Structural Correlates of Interactions of Hydrophobic Polyamine Endotoxin Sequestrants with Human Serum Albumin. *Mol Pharm (epublication).*
Abstract: Hydrophobically substituted polyamine compounds, particularly N-acyl or N-alkyl derivatives of homospermine, are potent endotoxin (lipopolysaccharide) sequestrants. Despite their polycationic nature,

the aqueous solubilites are limited owing to the considerable overall hydrophobicity contributed by the long-chain aliphatic substituent, but solubilization is readily achieved in the presence of human serum albumin (HSA). We desired first to delineate the structural basis of lipopolyamine-albumin interactions and, second, to explore possible structure-activity correlates in a well-defined, congeneric series of N-alkyl and -acyl homospermine lead compounds. Fluorescence spectroscopic and isothermal titration calorimetry (ITC) results indicate that these compounds appear to bind to HSA via occupancy of the fatty-acid binding sites on the protein. The acyl and carbamate compounds bind HSA the strongest; the ureido and N-alkyl analogues are significantly weaker, and the branched alkyl compound is weaker still. ITC-derived dissociation constants are weighted almost in their entirety by enthalpic Delta H terms, which is suggestive that the polarizability of the carbonyl groups facilitate, at least in large part, their interactions with HSA. The relative affinities of these lipopolyamines toward HSA is reflected in discernible differences in apparent potencies of LPS-sequestering activity under experimental conditions requiring physiological concentrations of HSA, and also of in vivo pharmacodynamic behavior. These results are likely to be useful in designing analogues with varying pharmacokinetic profiles

Panja S., Jana B., Aich P. and Basu T. (2008) In vitro interaction between calf thymus DNA and Escherichia coli LPS in the presence of divalent cation Ca2+. *Biopolymers* **89,** 606-613.
Abstract: With increasing addition of Escherichia coli LPS to calf thymus DNA, both dissolved in CaCl2, absorption maxima of DNA at 260 nm decreased gradually with the appearance of isosbastic points at both ends of spectra, which implied some binding between DNA and LPS. Hill plot of absorbance data showed that the binding interaction was positive cooperative in nature. For any fixed concentration of DNA and LPS, extent of interaction increased as concentration of CaCl2 was raised from 1.0 to 100 mM, signifying the electrostatic nature of the interaction, mediated through Ca2+ ion. Stepwise addition of EDTA, a chelating agent for divalent cations, to DNA-LPS bound complex gradually reversed the spectral shift with increase in absorbance at 260 nm, which implied opening up of the complex, that is, reversible nature of the interaction. Circular dichroism spectral changes of DNA by the addition of LPS indicated partial transition of DNA from B to A form. Isothermal titration calorimetric (ITC) study showed that the DNA-LPS binding was an exothermic and enthalpy-driven phenomenon. Moreover, in the presence of 100 mM CaCl2, binding constant of the interaction was found to be 2.6 x 10(4) M(-1) and 3.1 x 10(4) M(-1) from the analysis of Hill plot and ITC result, respectively. DNA-melting study showed that the LPS binding had increased the melting temperature of DNA, indicating more stabilization of DNA double helix. The binding of LPS to DNA made the complex resistant to digestion with endonucleases EcoRI and DNase I

Panja S., Aich P., Jana B. and Basu T. (2008) Plasmid DNA binds to the core oligosaccharide domain of LPS molecules of E. coli cell surface in the CaCl2-mediated transformation process. *Biomacromolecules.* **9,** 2501-2509.
Abstract: In the standard procedure for artificial transformation of E. coli by plasmid DNA, cellular competence for DNA uptake is developed by suspending the cells in ice-cold CaCl2 (50-100 mM). It is believed that CaCl2 helps DNA adsorption to the lipopolysaccharide (LPS) molecules on E. coli cell surface; however, the binding mechanism is mostly obscure. In this report, we present our findings of an in-depth study on in vitro interaction between plasmid DNA and E. coli LPS, using different techniques like absorption and circular dichroism spectroscopy, isothermal titration calorimetry, electron and atomic force microscopy, and so on. The results suggest that the Ca(II) ions, forming coordination complexes with the phosphates of DNA and LPS, facilitate the binding between them. The binding interaction appears to be cooperative, reversible, exothermic, and enthalpy-driven in nature. Binding of LPS causes a partial transition of DNA from B- to A-form. Finer study with the hydrolyzed products of LPS shows that only the core oligosaccharide domain of LPS is responsible for the interaction with DNA. Moreover, the biological significance of this interaction becomes evident from the observation that E. coli cells, from which the LPS have been leached out considerably, show higher efficiency of transformation, when transformed with plasmid-LPS complex rather than plasmid DNA alone

Perry T. D., Klepac-Ceraj V., Zhang X. V., McNamara C. J., Polz M. F., Martin S. T., Berke N., and Mitchell R. (2005) Binding of harvested bacterial exopolymers to the surface of calcite. *Environ Sci Technol* **39,** 8770-8775.
Abstract: Biologically produced exopolysaccharides (EPS) affect calcite dissolution and precipitation. In this study, natural alkaliphilic microbial isolates were collected from biofilms on historic limestone. The

isolates were screened for their ability to produce significant quantities of EPS in cultures. The most productive isolates were identified by 16S rRNA sequence analysis as a close relative of Bacillus cereus. EPS with different chemical structures were harvested from the isolates. Isothermal titration calorimetry (ITC) was used to quantify the thermodynamics of binding by the harvested EPS to calcite. The binding was described by a Langmuir adsorption isotherm. Characterization of the EPS showed that binding strength to calcite depended on the chemical nature of the polymer.

Sakai H., Hisamoto S., Fukutomi I., Sou K., Takeoka S., and Tsuchida E. (2004) Detection of lipopolysaccharide in hemoglobin-vesicles by Limulus amebocyte lysate test with kinetic-turbidimetric gel clotting analysis and pretreatment of surfactant. *J Pharm Sci* **93,** 310-321.
Abstract: A method to quantitatively measure the bacterial endotoxin content (lipopolysaccharide, LPS) in phospholipid vesicles or liposomes is necessary because the conventional Limulus amebocyte lysate (LAL) test does not provide an accurate measurement due to the hydrophobic interaction of LPS and vesicles that shields the activity of LPS to clot the LAL coagulant. This interference was evident from isothermal titration calorimetry results in our study that clearly demonstrated the insertion of the LPS molecule into the phospholipid bilayer membrane. Hemoglobin-vesicles (HbVs; particle diameter = 251 +/- 80 nm; [Hb] = 10 g/dL) are artificial oxygen carriers encapsulating a conc. Hb solution in phospholipid vesicles, and their oxygen transporting ability has been extensively studied. To accurately measure the LPS content in the HbV suspension, we tested the solubilization of HbV with deca(oxyethylene) dodecyl ether (C(12)E(10)), used to release the LPS entrapped in the vesicles, as a pretreatment for the succeeding LAL assay of the kinetic-turbidimetric gel clotting (detecting wavelength, 660 nm). The C(12)E(10) surfactant interferes with the gel clotting in a concentration-dependent manner, and the optimal condition was determined in terms of minimizing the dilution factor and C(12)E(10) concentration. We clarified the condition that allowed the measurement of LPS at >0.1 endotoxin units (EU)/mL in the HbV suspension. Moreover, the utilization of histidine-immobilized agarose gel effectively concentrated the trace amount of LPS from the C(12)E(10)-solubilized HbV solution and washed out C(12)E(10) as an inhibitory element. The LAL assay with the LPS-adsorbed gel resulted in the detection limit of 0.0025 EU/mL. Pretreatment with C(12)E(10) would be applicable not only to HbVs but also to other drug delivery systems using phospholipid vesicles encapsulating or incorporating functional molecules.

Yin N., Marshall R. L., Matheson S., and Savage P. B. (2003) Synthesis of lipid A derivatives and their interactions with polymyxin B and polymyxin B nonapeptide. *J Am Chem Soc* **125,** 2426-2435.
Abstract: Lipid A is the causative agent of Gram-negative sepsis, a leading cause of mortality among hospitalized patients. Compounds that bind lipid A can limit its detrimental effects. Polymyxin B, a cationic peptide antibiotic, is one of the simplest molecules capable of selectively binding lipid A and may serve as a model for further development of lipid A binding agents. However, association of polymyxin B with lipid A is not fully understood, primarily due to the low solubility of lipid A in water and inhomogeneity of lipid A preparations. To better understand lipid A-polymyxin B interaction, pure lipid A derivatives were prepared with incrementally varied lipid chain lengths. These compounds proved to be more soluble in water than lipid A, with higher aggregation concentrations. Isothermal titration calorimetric studies of these lipid A derivatives with polymyxin B and polymyxin B nonapeptide indicate that binding stoichiometries (peptide to lipid A derivative) are less than 1 and that affinities of these binding partners correlate with the aggregation states of the lipid A derivatives. These studies also suggest that cooperative ionic interactions dominate association of polymyxin B and polymyxin B nonapeptide with lipid A.

Zuckermann M. J. and Heimburg T. (2001) Insertion and pore formation driven by adsorption of proteins onto lipid bilayer membrane-water interfaces. *Biophys J* **81,** 2458-2472.
Abstract: We describe the binding of proteins to lipid bilayers in the case for which binding can occur either by adsorption to the lipid bilayer membrane-water interface or by direct insertion into the bilayer itself. We examine in particular the case when the insertion and pore formation are driven by the adsorption process using scaled particle theory. The adsorbed proteins form a two-dimensional "surface gas" at the lipid bilayer membrane-water interface that exerts a lateral pressure on the lipid bilayer membrane. Under conditions of strong intrinsic binding and a high degree of interfacial converge, this pressure can become high enough to overcome the energy barrier for protein insertion. Under these conditions, a subtle equilibrium exists between the adsorbed and inserted proteins. We propose that this provides a control mechanism for reversible insertion and pore formation of proteins such as melittin and magainin. Next, we

discuss experimental data for the binding isotherms of cytochrome c to charged lipid membranes in the light of our theory and predict that cytochrome c inserts into charged lipid bilayers at low ionic strength. This prediction is supported by titration calorimetry results that are reported here. We were furthermore able to describe the observed binding isotherms of the pore-forming peptides endotoxin (alpha 5-helix) and of pardaxin to zwitterionic vesicles from our theory by assuming adsorption/insertion equilibrium.