

Kinetics of the interaction of endotoxin with polymyxin B and its analogs: a surface plasmon resonance analysis

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Abstract Lipopolysaccharide, the invariant structural component of Gram-negative bacteria, when present in minute amounts in the circulation in humans elicits 'endotoxic shock' syndrome, which is fatal in 60% of the cases. Polymyxin B (PMB), a cyclic cationic peptide, neutralizes the endotoxin, but also induces many harmful side effects. Many peptide-based drugs mimicking the activity of PMB have been synthesized in an attempt to reduce toxicity while still retaining the anti-endotoxic activity. The study attempts to use the recent technique of surface plasmon resonance (SPR), in determining the kinetics of association and dissociation involved in the interaction of endotoxin with a few selected peptides that have structural features resembling PMB. The results, in conjunction with the thermodynamic data derived using isothermal titration calorimetry (ITC), stress the vital role played by amphiphilicity of the peptides and hydrophobic forces in this biologically important interaction.

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Key words: Lipopolysaccharide; Polymyxin B; Surface plasmon resonance; Isothermal titration calorimetry; Kinetics; Amphiphilicity

1. Introduction

Lipopolysaccharide (LPS), the invariant structural component of the Gram-negative bacterial outer membranes [1–5], elicits numerous pleiotropic effects on susceptible cells/organisms including B-cell mitogenesis, activation of viruses, complement and cytokine cascade, mimicry of acute graft versus host disease, pyrogenicity and lethality in mammals and triggering of the defense and clotting cascade in the horseshoe crab [6,7]. The most dreaded sequel of LPS in the circulation, caused by infection with Gram-negative bacteria, is termed 'endotoxic shock'. Endotoxic shock is characterized by hemodynamic and coagulation abnormalities [8], culminating in the multiple system organ failure which accounts for about 60% fatalities in humans [9]. All of the toxic and pleiotropic activities of LPS are confined to its lipid A moiety (an acylated phosphodisaccharide unit [10,11]). Removal of LPS by molecules that interact strongly and specifically constitutes an important stratagem for combating endotoxic shock [12–21].

Polymyxin B (PMB) and related family members of cyclic cationic decapeptide antibiotics obtained from *Bacillus polymyxa* are used for treating severe cases of sepsis [22–24]. However, the usage of PMB in the prophylaxis of sepsis is limited on account of its toxicity due to the harmful side effects associated with its slow degradation in vivo, attributed to the presence of uncommon D-amino acids, and its unusual covalent

structure [25,26]. We have previously shown that the amphiphilicity of PMB is both necessary and adequate for its stoichiometric interaction with LPS [27,28]. As the analyses of the kinetics and mechanism of macromolecule-ligand interaction provides valuable insights into the biomolecular recognition processes, we report here on the surface plasmon resonance analyses of the reaction of PMB, polymyxin B nonapeptide (PMBN), as well as two synthetic cyclic peptide analogs of PMB, with the endotoxin molecules. These studies attempt to draw structure-activity correlation between them and show that the amphiphilic distribution of the amino acid side chains in them plays a crucial role in their recognition of LPS [29–32].

2. Materials and methods

2.1. Materials

LPS from *Escherichia coli* strains 055:B5, obtained from Sigma Chemical Co. (St. Louis, MO, USA), was repurified as described earlier by Srimal et al. [27]. Diphosphoryl lipid A (heptacyl) derived from *E. coli* was from List Biologicals, PMB and PMBN were obtained from Sigma. All the other chemicals used were of the highest purity available.

2.2. Peptide synthesis

The cyclic hepta- and decapeptide were synthesized on a solid phase peptide synthesizer (NovaSyn) using standard Fmoc and Opfp chemistry. The peptides were cleaved from *p*-hydroxymethylphenoxymethylpolystyrene resin using 1,2-ethanedithiol (0.15 ml), deionized water (0.5 ml) and trifluoroacetic acid (10 ml). The sulfhydryl bridge in the decapeptide was introduced according to the method of Wu et al. [33]. They were purified by reverse phase HPLC on a C-18 (Vydac, USA) column using a water/acetonitrile gradient. Their purity and molecular weight were checked by matrix assisted laser desorption ionization (MALDI) spectroscopy.

2.3. Preparation of LPS solution

LPS suspended in 50 mM phosphate buffer pH 6.8 was vortexed vigorously for 10 min at 70°C and mixed with equimolar concentration of triethylamine with respect to the anionic groups in them. Samples were sonicated for 5 min using a Braun probe sonicator prior to use.

2.4. Quantification of LPS/lipid A and PMB

LPS samples were quantified using the endotoxin from Endosafe, as the standard, by the *Limulus* amoebocyte lysate assay in pyrogen-free water according to the method of Yin et al. [34], prior to all the assays. PMB was quantified by its molar absorbance [27].

2.5. Surface plasmon resonance (SPR) analysis

Binding kinetics were determined by SPR using a BIAcore 2000 biosensor system. The peptides were covalently immobilized on the certified grade CM5 sensor chips at concentrations of 40 µg/ml in 10 mM sodium acetate, pH 4.8, using the amine coupling kit supplied by the manufacturer. Nearly 500 resonance units (RU) of peptide were immobilized under these conditions, where 1 RU corresponds to immobilized peptide concentration of ~1 pg/mm². The unreacted moieties on the surface were blocked with ethanolamine. All measurements were carried out in 10 mM HEPES, pH 7.4, 150 mM NaCl,

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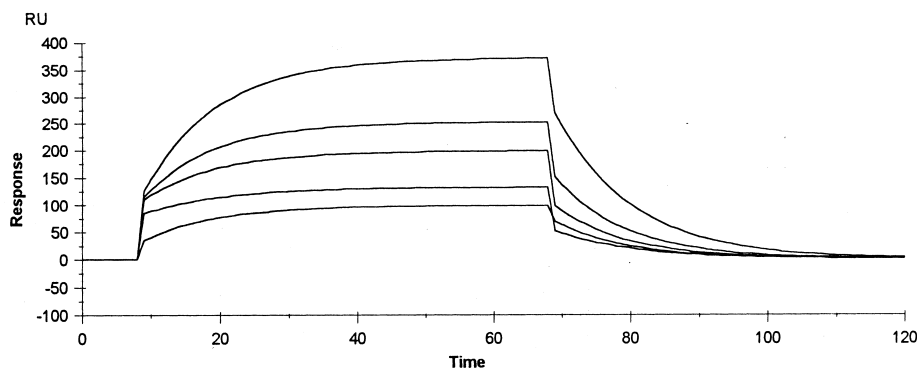


Fig. 1. Overlay plot of sensograms depicting the interaction of LPS with immobilized cyclic decapeptide. LPS ranging in concentration from 25 to 125 nM was injected for 50 s at a flow rate of 10 $\mu\text{l}/\text{min}$ and concentrations from bottom to top: 25, 50, 75, 100 and 125 nM. After the dissociation phase, by passing PMB (1 mM) in a running buffer, at 10 $\mu\text{l}/\text{min}$, the surface was regenerated by a pulse of 10 mM NaOH at a flow rate of 10 $\mu\text{l}/\text{min}$. Restrictions in the mobilities of the fluorescent probe diphenylhexatriene showed that the LPS used in the studies was confirmed to be in the lamellar phase [28,43].

3.4 mM EDTA. For the determination of association rate constants (k_1), LPS (25–125 nM), flowing at a rate of 10 $\mu\text{l}/\text{min}$, in the same buffer was used. Dissociation rate constants (k_{-1}), were evaluated by passing a solution of 1 mM PMB, in the same buffer at a flow rate of 10 $\mu\text{l}/\text{min}$. The surface was regenerated by a 10-s pulse of 10 mM NaOH flowing at 50 $\mu\text{l}/\text{min}$.

2.5.1. Data analysis. Rate constants k_1 and k_{-1} were obtained by fitting the primary sensogram data using the BIA evaluation 3.0 software. The dissociation rate constant is derived using equation 1:

$$R_t = R_{t0} e^{-k_{-1}(t-t_0)} \quad (1)$$

where the R_t is the response at time t , R_{t0} is the amplitude of the initial response, and k_{-1} is the dissociation rate constant. The association rate constant k_1 can be derived using equation 2, from the measured k_{-1} values:

$$R_t = R_{\text{Max}} [1 - e^{-(k_1 C + k_{-1})(t-t_0)}] \quad (2)$$

where R_t is the response at time t , R_{Max} is the maximum response, C is the concentration of the analyte in the solution, k_1 and k_{-1} are the association and dissociation rate constants respectively. The ratio of k_1 and k_{-1} yields the value of association constant K_a (k_1/k_{-1}).

All ITC experiments were performed as previously reported by Srimal et al. [27], using an OMEGA high sensitivity microcalorimeter, manufactured by MicroCal Inc [35]. A typical titration involved 15–20 injections at 3 min intervals, 4 μl aliquots of peptide solution into the sample cell (volume 1.344 ml) containing LPS (50 mM). The titration cell was stirred continuously at 400 rev/min. The molecular mass of LPS was taken to be 20000 Da [27]. The heats of the dilution of the peptides in the buffer alone were subtracted from the titration data.

The resulting data were then analyzed to determine the binding stoichiometry (n), association constant and the enthalpy change (ΔH_b) as described earlier. ΔG_b^0 and ΔS_b^0 were calculated from the fundamental equation of thermodynamics:

$$\Delta G_b^0 = -RT \ln K_b \quad (3)$$

$$\Delta S_b^0 = (\Delta H_b - \Delta G_b^0)/T \quad (4)$$

3. Results

A typical sensogram for the binding of the varying concentrations of cyclic peptide immobilized on the CM5 sensor chip is shown in Fig. 1. Occurrence of a rapid enhancement in the RUs on the association of LPS to the cyclic peptide with time reflects the accompanying changes in the mass during the reaction. The analyses of the sensogram data gives k_1 and k_{-1} for the interaction of LPS with the cyclic peptide as $3.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and 0.092 s^{-1} respectively. The distribution of the residuals indicated that the time dependence of RUs for both the association and the dissociation reactions is fitted satisfactorily to a monoexponential reaction. Non-binding of LPS to the dextran matrix attests to the specificity of the interactions of the peptides with LPS (Fig. 2, d). The association of the LPS to peptides shows that it binds to PMB with not only the

Table 1
Kinetic parameters of LPS binding to peptides at 20°C surface plasmon resonance analysis

Peptide	Salt (M)	$k_1 \times 10^{-4} (\text{M}^{-1} \text{ s}^{-1})$	$k_{-1} (\text{s}^{-1})$	$K_a \times 10^{-5} (\text{M}^{-1})$	$-\Delta G^0 (\text{kJ mol}^{-1})$
PMB	0.0	11.0 (11.9)	0.070	15.71	34.74
	0.25	10.5 (11.1)	0.070	15.00	34.63
	1.00	9.3 (9.9)	0.070	13.28	34.34
PMBN	0.0	3.2 (3.5)	0.083	3.85	31.33
	0.25	3.1 (3.4)	0.082	3.78	31.03
	1.00	2.8 (2.9)	0.082	3.41	31.08
Cyclic decapeptide	0.0	5.2 (5.0)	0.090	5.77	32.31
	0.25	5.0 (4.7)	0.090	5.55	32.22
	1.00	4.8 (4.2)	0.090	5.05	31.99
Cyclic ^a heptapeptide	—	0.2	≈ 1	≈ 0.02	18.5

Dansyl-PMB-LPS interaction by stopped flow fluorimetry gives k_1 , k_2 , k_{-1} , k_{-2} $1.98 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, 0.341, 0.458, 0.0571 s^{-1} respectively. The overall binding constant K_a ($[k_1/k_{-1}]$, $[k_2/k_{-2}]$) is $2.5 \times 10^6 \text{ M}^{-1}$ which is slightly higher than the value of K_a obtained by the SPR method. Dansyl-PMBN-LPS interaction by stopped-flow fluorimetry shows only single phases ($k_1 = 3.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{-1} = 0.125 \text{ s}^{-1}$ association and dissociation s^{-1}). Value of k_1 in parentheses indicates analysis of sensograms, using mass transport limited kinetics yields the same results. Values of k_{-1} were essentially the same.

^aIndicates binding was too weak to be determined accurately.

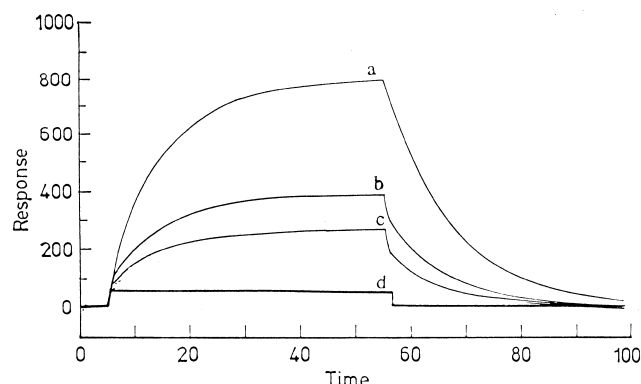


Fig. 2. Comparison of SPR derived binding curves for different immobilized peptides interacting with LPS. LPS at fixed concentration (125 nM, except for cyclic heptapeptide, where the concentration used was 500 μ M) was injected for 50 s at a flow rate of 10 μ L/min. The sensograms represent binding of, from bottom to top: cyclic heptapeptide, PMBN, cyclic decapeptide, PMB and to blank control chip. The association (k_1) and dissociation rate constants (k_{-1}) were derived by fitting the sensograms to a Langmuir binding rate equations (Eqs. 1 and 2) and are tabulated in Table 1.

highest affinity but also with greatest rapidity (Fig. 2, a). Moreover, the residence time of LPS on PMB was longer than that of other peptides (Table 1). The association rates of the interaction of LPS to the peptides, excepting the cyclic heptapeptide, range from $3.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ to $3.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, while the dissociation rates are 0.086–0.0987 s^{-1} . The cyclic heptapeptide has abysmally low k_1 ($\approx 2 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) and high k_{-1} ($\geq 1 \text{ s}^{-1}$). The dissociation rate constants were also determined by the plot of $\ln(R_1/R_t)$ versus time. The values of the k_{-1} thus obtained were 0.083, 0.098, 0.092 s^{-1} for PMB, PMBN and the cyclic peptide, respectively and were similar to those obtained by fitting the original plots. The effects of varying salt concentrations on the kinetics of LPS-PMB interaction are also shown in Table 1. Mass transport limited kinetic analysis of these sensograms yielded similar rate constants (Table 2, Fig. 3).

Isothermal titration calorimetry for the binding of these peptides was conducted to obtain an independent measure of their affinities, binding enthalpies as well as the changes in the heat capacities. The binding constant (K_a) for cyclic decapeptide, PMBN and cyclic heptapeptide are 3.5×10^5 , 2.1×10^5 , $2 \times 10^3 \text{ M}^{-1}$ respectively which are in excellent agreement with the value obtained by SPR analyses. The values of K_a for PMB-LPS interaction are close to those obtained by an earlier ITC study. All the peptides show endothermic changes in enthalpies which lie in the range 24–12 kJ/mol as well as negative changes in heat capacities (ΔC_p). However, the ΔH and ΔC_p values for the cyclic heptapeptide could not be determined due to its poor affinity for the endotoxin (Table 1).

4. Discussion

SPR is a rapid method for evaluating the elementary step involved in the interaction between a macromolecule and its complementary ligand as well as the affinities involved therein as proven by a wealth of data in the literature [36–41]. As the method relies exclusively on the mass change, the biomolecular interaction can be studied in real time without taking recourse to any external labels such as radioisotopes or fluo-

rophores which, in some instances, can alter the nature of the reaction. Moreover, SPR is perhaps the only technique that can provide data for both the association and dissociation phases of a reaction in a single experimental run.

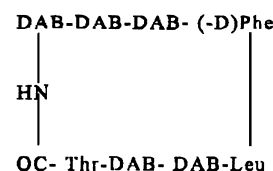
These studies are based on the premise that characterization of the interaction of PMB and related peptides to LPS could lend to an understanding of the mechanism of their interaction with endotoxin. It is therefore, pertinent to relate the structure of PMB and related peptides used in this study with their LPS interaction abilities, which are depicted below.

Polymyxin B

X- DAB-Thr-DAB-DAB-DAB- (-D)Phe



Nonapeptide



Cyclic decapeptide



Cyclic heptapeptide



where X- corresponds to 6-heptanoyl/octanoyl moieties attached at the N-terminus of PMB and DAB. Cyclic deca- and heptapeptide are represented by single letter code and are based on the work of Porro et al. [25].

Using stopped-flow fluorescence spectrometry we have recently reported that PMB-LPS interaction consists of a pair of kinetically distinguishable on and off reactions [28]. On the other hand PMBN-LPS interactions exhibit monoexponential association and dissociation rates (unpublished observations). The SPR data show that PMB and the other peptides interact with LPS in a monoexponential manner. This is not surprising as SPR measures mass changes alone as a function of time. Consequently, subtle conformational changes involved in the macromolecular-ligand interactions are difficult to observe. The values of k_1 and k_{-1} for the binding of PMB-LPS interactions estimated from the SPR experiment are close to those determined by the stopped-flow spectrofluorimetry for the faster phase of the reaction. However, as the PMBN as well as other peptides display a monoexponential binding behavior only and the SPR values of k_1 and k_{-1} for PMB-LPS interactions are notably similar to those of the fast phase of the reaction evaluated using the former method. These data taken together with the fact that the contribution of the slower phase of the reaction for PMB-LPS interaction is small to the overall binding constant, it is adequate to compare the

Table 2
Thermodynamic parameters for LPS binding to peptides at 20°C

Peptide	<i>T</i> (°C)	<i>n</i>	$K_a \times 10^3$ (M ⁻¹)	ΔH_b (kJ mol ⁻¹)	$-\Delta G^0$ (kJ mol ⁻¹)	ΔS_b (J mol ⁻¹ K ⁻¹)	$-\Delta C_p$ (J mol ⁻¹ deg ⁻¹)
PMB	20	0.93	1205	24.2	34	198	2440
	25	1.02	3250	12.1	37	164	
PMBN	20	0.98	310	15.9	30	156	1160
	25	1.09	725	10.1	33	144	
Cyclic decapeptide	20	1.05	580	20.2	32	184	1640
	25	1.06	960	12.0	34	154	
Cyclic ^a heptapeptide	—	0.63	2.7	—	—	—	—

^aIndicates that the data obtained are subject to error greater than 10%.

binding properties of the peptides using the SPR analysis. Inspection of the binding parameters determined in the SPR experiments show that the endotoxin molecule binds to PMB the strongest, due to a faster on as well as a slower off rate. While LPS also shows a similar k_1 for the binding to cyclic decapeptide its faster dissociation perhaps accounts for its relatively lower affinity. The poorer affinity for PMBN for LPS is accounted for by a relatively slower k_1 and a faster k_{-1} as compared to PMB. The cyclic heptapeptides extremely poor affinity for LPS is largely due to its slow k_1 and the very fast k_{-1} reactions ($k_1 = 2 \times 10^3$ M⁻¹ s⁻¹, $k_{-1} \geq 1$ s⁻¹) as compared to PMB.

The values of binding constants for the interaction of these peptides with isothermal titration calorimetry experiments are in close agreement with those from SPR analyses. This indi-

cates that the SPR experiments are reporting a total binding event. Moreover, the ITC data show that the binding of the peptides to LPS is driven by hydrophobic forces [28]. The kinetics of binding of LPS with PMB and related peptides appears to be dominated by van der Waals and hydrophobic interactions as, even at 1 M salt concentration where the electrostatic interactions can be considered to be completely neutralized, both the on and the off rates are affected marginally [27]. Therefore, the electrostatic interaction between LPS and PMB, perhaps plays a minor role during the reaction. Thus the role of the positively charged DAB side chains of PMB and other analogous peptides is largely related to their ability to maintain the amphiphilicity of these peptides.

In an earlier study we had proposed that the amphipathic distribution, viz. the asymmetric location of the positively charged polar and the non-polar amino acid side chains at disparate regions are responsible for its strong and stoichiometric binding to LPS [27,28,42]. That amphiphilicity is both necessary and sufficient for the interaction of the peptides to LPS is proven further by the binding of LPS to a synthetic peptide with a vectorial segregation of polar and non-polar amino acid side chains but which bears no structural resemblance to PMB [25]. Since PMBN lacks the acyl chain at its amino-terminus, it has poorer affinity and kinetics of binding to LPS which is related to its relatively lower amphiphilicity as compared to that of PMB. The k_1 for the binding of LPS to the cyclic decapeptide is comparable to that of PMB. This is not surprising as its amphiphilicity is higher when compared to PMBN due to the presence of lysyl side chains which contains two extra methylene groups in place of the DAB side chains of the latter. An extremely poor kinetics and affinity of binding of LPS to the cyclic heptapeptide which does not display any amphiphilicity proves further that amphiphilicity is both necessary and sufficient for the binding of peptides to LPS.

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References

- [1] Lieve, L. (1974) *Ann. NY Acad. Sci.* 235, 109–129.
- [2] Sanderson, K.E., MacAlister, T.J. and Costerton, J.W. (1974) *Can. J. Microbiol.* 14, 239–334.
- [3] Galanos, C., Luderitz, O., Rietschel, E.T. and Westphal, O. (1977) *Int. Rev. Biochem.* 14, 239–334.
- [4] Westphal, O. (1975) *Trans. Coll. Int. Aller.* 49, 1–43.
- [5] Raetz, C.R.H. (1990) *Annu. Rev. Biochem.* 59, 129–170.

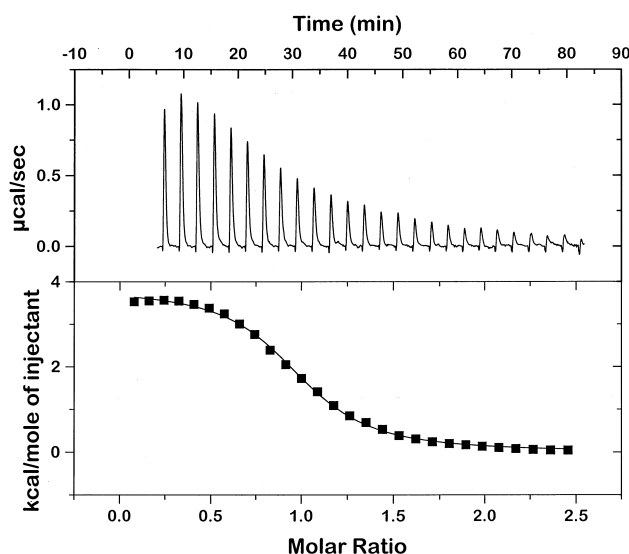


Fig. 3. Calorimetric titration for the binding of PMBN to LPS. (*E. coli* 055:B55) Enthalpies were determined by titration of 1.5 mM of PMBN into 0.05 mM of LPS in 50 mM sodium phosphate buffer (pH 6.8) at 20°C in a OMEGA titration calorimeter (MicroCal, Northampton, MA, USA). LPS solution was prepared as described in the text and its molecular weight was taken to be 20 000 Da. The cell was thermostatically controlled to $\pm 0.1^\circ\text{C}$ with a circulating bath. a: Twenty-seven 4 μl injections were made into LPS and the heat exchange measured. Injections occurred over 5 s at 3 min intervals. Incremental heats of binding in reaction to the injection number are shown in panel b. The data were fitted by the non-linear least squares minimization method subsequent to the correction for the heats of dilution for the peptide which was small. The thermodynamic parameters of other peptides are shown in Table 2.

- [6] Reitschel, E.T. and Brade, H. (1992) *Sci. Am.* 7, 26–33.
- [7] Iwanaga, S. (1993) *Curr. Opin. Immunol.* 1, 74–84.
- [8] Fink, P.F. (1990) in: *Sepsis Syndrome. Handbook of Critical Care*, 3rd edn. (Berk, J.L. and Sampliner, J.E., Eds.), p. 619, Little, Brown and Co., Boston, MA.
- [9] Zeigler, E.T. et al. (1991) *New Engl. J. Med.* 324, 429–436.
- [10] Reitschel, E.T. and Brade, H. (1992) *Sci. Am.* 7, 26–33.
- [11] Neter, E.O., Westphal, O., Luderitz, O., Gorzynski, E.A. and Cohenberger, E. (1956) *J. Immunol.* 11, 377–385.
- [12] Cross, A.S. and Opal, S. (1994) *J. Endotoxin Res.* 1, 57–69.
- [13] Dentener, M.A. et al. (1993) *J. Immunol.* 151, 4258–4265.
- [14] Elsbach, P. and Weiss, J. (1993) *J. Immunobiol.* 187, 417–429.
- [15] Fisher Jr, C.J., Marra, M.A., Palardy, J.E. and Marchbanks, C.R. (1994) *Crit. Care Med.* 22, 553–558.
- [16] Nelson, D., Kuppermann, N. and Fleisher, G.R. et al. (1995) *Crit. Care Med.* 23, 92–98.
- [17] Christ, W.J., Asano, O. and Robidoux, A.L.C. et al. (1995) *Science* 268, 80–83.
- [18] Kirikae, T., Schade, F.U., Kirikae, F., Qureshi, N., Takayama, K. and Reitschel, E.T. (1994) *FEMS Immunol. Med. Microbiol.* 9, 237–244.
- [19] Loppnow, H., Libby, P., Freudenberg, M., Kruss, J.H., Weckesser, J. and Mayor, H. (1990) *Infect. Immun.* 58, 3743–3750.
- [20] Novogrodsky, A., Vanichkin, A., Patya, M., Gazit, A., Oshero, N. and Levitzki, A. (1994) *Science* 264, 1319–1322.
- [21] Quezado, Z.M.N., Banks, S.M. and Natanson, C. (1995) *Trends Biotechnol.* 13, 56–63.
- [22] Morrison, D.C. and Jacobs, D.M. (1976) *Immunochemistry* 13, 813–818.
- [23] Schindler, M. and Osborn, M.J. (1979) *Biochemistry* 18, 4425–4430.
- [24] Schindler, M. and Teuber, M. (1975) *Antimicrob. Agents Chemother.* 8, 95–104.
- [25] Rustici, A., Velucchi, M., Faggioni, R., Sironi, M., Ghezzi, P., Quataert, S., Green, B. and Porro, M. (1993) *Science* 259, 361–365.
- [26] Storm, D.R., Rosenthal, K.S. and Swanson, P.E. (1977) *Annu. Rev. Biochem.* 46, 723–763.
- [27] Srimal, S., Suroli, N., Balasubramaian, S. and Suroli, A. (1996) *Biochem. J.* 315, 679–686.
- [28] Thomas, C.J., Gangadhar, B.P., Suroli, N. and Suroli, A. (1998) *J. Am. Chem. Soc.* 120, 12428–12434.
- [29] Schroder, G., Brandenburg, K. and Seydel, U. (1992) *Biochemistry* 31, 631–638.
- [30] Hartmann, W., Galla, H.J. and Sackmann, E. (1978) *Biochim. Biophys. Acta* 510, 124–139.
- [31] Chapman, T.M. and Golden, M.R. (1972) *Biochem. Biophys. Res. Commun.* 46, 2040–2047.
- [32] Galardy, R.E., Craig, L.C. and Printz, M.P. (1974) *Biochemistry* 12, 164–177.
- [33] Lio, S.-Y., Ong, G.-T., Wang, K.-T. and Wu, S.-H. (1995) *Biochim. Biophys. Acta* 1252, 312–320.
- [34] Yin, E.T., Galanos, C., Kinsky, S., Bradshaw, R.A., Wessler, S., Luderitz, O. and Sarmiento, M.E. (1972) *Biochem. Biophys. Acta* 261, 284–288.
- [35] Wiseman, T., Williston, S., Brandts, J.F. and Lin, L.N. (1989) *Anal. Biochem.* 179, 131–137.
- [36] Vadgama, P. and Crump, P.W. (1992) *Analyst* 117, 1657–1670.
- [37] Jönsson, U., Fägerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Löfas, S., Persson, B., Roos, H. and Rönnerberg, I. (1991) *BioTechniques* 11, 620–627.
- [38] O'Shannessy, D.J. (1994) *Curr. Opin. Biotechnol.* 5, 65–71.
- [39] Szabo, A., Stolz, L. and Granzow, R. (1995) *Curr. Opin. Struct. Biol.* 5, 699–705.
- [40] Brigham-Burke, M., Edwards, J.R. and O'Shannessy, D.J. (1992) *Anal. Biochem.* 205, 125–131.
- [41] O'Shannessy, D.J., Brigham-Burke, M. and Peck, K. (1992) *Anal. Biochem.* 205, 132–136.
- [42] Vara, M. and Vara, T. (1983) *Nature* 309, 526–528.
- [43] Seydel, U., Brandenburg, K., Koch, M.H.J. and Reitschel, E.T. (1989) *Eur. J. Biochem.* 186, 325–332.