Anti-Endotoxin Agents. 1. Development of a Fluorescent Probe Displacement Method Optimized for the Rapid Identification of Lipopolysaccharide-Binding Agents

Stewart J. Wood, Kelly A. Miller and Sunil A. David*

Department of Medicinal Chemistry, University of Kansas, Life Sciences Research Laboratories, 1501 Wakarusa Drive, Lawrence, KS – 66049, USA

Abstract: Lipopolysaccharides (LPS), otherwise termed 'endotoxins', are outer-membrane constituents of Gram-negative bacteria. Lipopolysaccharides play a key role in the pathogenesis of 'Septic Shock', a major cause of mortality in the critically ill patient. Therapeutic options aimed at limiting downstream systemic inflammatory processes by targeting lipopolysaccharide do not exist at the present time. We have defined the pharmacophore necessary for small molecules to specifically bind and neutralize LPS, and have shown using animal models of sepsis that the sequestration of circulatory LPS by small molecules is a therapeutically viable strategy. Assays reported previously in the literature do not lend themselves well to the rapid screening of large numbers of structurally diverse compounds. In this report, we describe a highly sensitive and robust fluorescent displacement assay using BODIPY TR cadaverine (BC), which binds specifically to the toxic center of LPS, lipid A, and is competitively displaced by compounds displaying an affinity for lipid A. The assay clearly discriminates subtle differences in the binding of polymyxin B, and its nonapeptide derivative, with LPS. The spectral properties of the BODIPY fluorophore are ideally suited for screening diverse structural classes of compounds, including those with conjugated aromatic groups, or with chromophores in the 260-500 nm range. The fluorescent probe: LPS complex is stable under physiologically relevant salt concentrations, resulting in the rapid rejection of spurious binders interacting *via* non-specific electrostatic interactions, and, therefore, in greatly improved dispersion of ED_{50} values.

Keywords: Endotoxin, Lipopolysaccharide, Sepsis, shock, Fluorescence, High-throughput screening.

INTRODUCTION

Gram-negative sepsis, or "blood poisoning" in lay terminology, is the thirteenth leading cause of overall mortality [1], and the number one cause of deaths in the intensive care unit [2] accounting for more than 200,000 fatalities in the US annually (Fig. **(1)**) [3]. Despite tremendous strides in antimicrobial chemotherapy, the incidence of sepsis has risen almost three-fold from 1979 through 2000 [4], and sepsis-associated mortality has essentially remained unchanged at about 45% [5] emphasizing a clear, unmet need to develop therapeutic options specifically targeting the pathophysiology of sepsis.

Lipopolysaccharide (LPS), otherwise termed 'Endotoxin' (signifying that the toxic is intrinsic to the bacterium, and not externally secreted or elaborated), is the major constituent of the outer leaflet of the outer membrane of all gram-negative bacteria [6-8]. Lipopolysaccharides, as the name suggests, consist of a highly variable and biologically inert polysaccharide portion and a structurally conserved, and toxically active lipid called lipid A [9, 10]. Lipid A, the active moiety of LPS [11], is composed of a hydrophilic, *bis*-phosphorylated (*bis*-anionic) diglucosamine backbone, and a hydrophobic domain of 6 (*E. coli*) or 7 (*Salmonella*) acyl chains in amide and ester linkages [12-14] (Fig. (**2**)).

The presence of LPS in blood (endotoxemia) sets off a cascade of exaggerated systemic inflammatory host response [15] that ultimately manifests clinically in the frequently fatal shock syndrome characterized by endothelial damage, coagulopathy, loss of vascular tone, myocardial dysfunction, tissue hypoperfusion, and multiple-system organ failure [16-18]. Underlying the overwhelming inflammatory response is the activation of the innate immune system [19, 20], which results in the production of a plethora of proinflammatory mediators, important among which are the cytokines tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and IL-6, secreted mainly by monocytes and macrophages (Mϕ) [21-24].

A logical approach to addressing therapeutically the problem of Gram-negative sepsis is to target lipopolysaccharide itself by the use of an agent that would bind to the lipid A moiety and sequester it, thereby preventing its recognition by LPS receptors [25-32] on the monocyte/macrophage and other effector cells. We have elected to systematically explore the approach of "proximal intervention" with the view that, in many respects, this would be preferable to alternate approaches directed at distal, downstream events (e.g. therapeutic targeting of the inflammatory mediators [31, 32], for once the monocyte/macrophage cell is activated, the cellular response is so diverse that a single pharmacological agent would be unlikely to modulate the effects of all the mediators produced).

The anionic and amphiphilic nature of lipid A (Fig. (**2**)) enables it to bind to numerous substances that are positively charged and also possess amphipathic character. We have, over the past decade, characterized the interactions of lipid A

*Address correspondence to this author at the 145E Bldg B, University of Kansas Life Sciences Research Laboratories, 1501 Wakarusa Drive, Lawrence, KS – 66049, Tel: 785-330-4316; Fax: 785-330-4332; E-mail: sdavid@ku.edu

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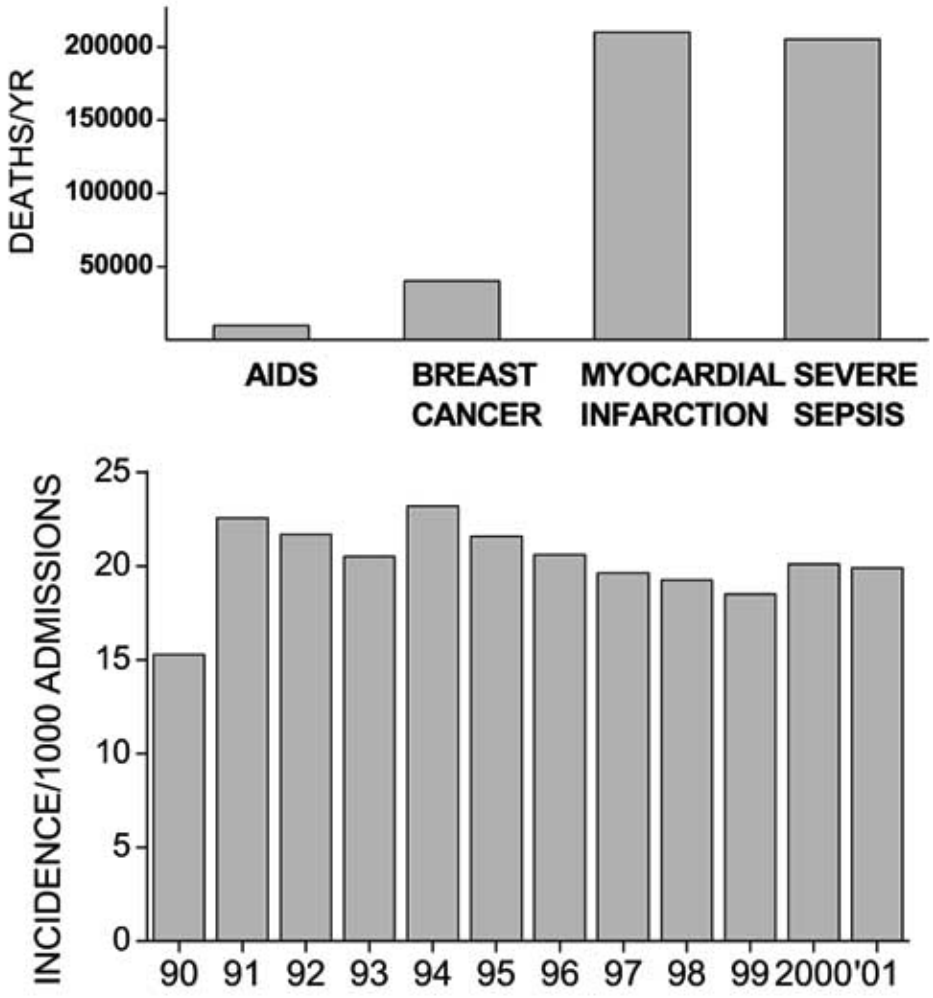

Fig. (1). *Top*: nationwide mortality due to sepsis. *Bottom*: Incidence of septic shock (ICD9CM Code: 785.59) at the University of Kansas Medical Center.

with a number of classes of cationic amphipathic molecules including proteins [33, 34], peptides [35-39], pharmaceutical compounds [40, 41], and other synthetic polycationic amphiphiles [42-45]. Importantly, from these and currently ongoing studies, we have determined the pharmacophore necessary for optimal recognition and neutralization of lipid A [41] by small molecules (for a recent review, see Ref. [46]).

Central to the identification of the pharmacophore was the development of a fluorescence displacement probe method using monodansylcadaverine (DC) [47], which afforded detailed analyses of the precise structural requirements in *bis*-cationic amphipathic molecules necessary for lipid A binding. In our continuing efforts to rapidly and quantitatively screen very large numbers of commercially-available compounds that fulfill the pharmacophore criteria for lipid A binding, we have found that the dansyl fluorophore is inadequate for the following reasons: (a) several classes of compounds of potential interest bear conjugated aromatic systems whose absorption confounds dansyl fluorescence *via* "inner-filter" effects; (b) in addition to emission intensity changes, the emission wavelength shifts as well when the probe binds to (or is displaced from) lipid A [41, 47] necessitating full emission scans on every well in a microtiter plate, thus greatly reducing the throughput of the assay; (c) the binding of DC to lipid A is relatively weak [47], and is primarily electrostatically driven, which greatly attenuates fluorescence changes under physiologically relevant salt concentrations; (d) the interaction of DC with native LPS is much weaker than that with lipid A, and it would be preferable to design a primary screen that utilizes LPS, rather than lipid A, since it is native LPS that is the clinically relevant target, and all subsequent biological assays on leads utilize LPS.

We now describe a highly sensitive and robust fluorescent displacement assay using BODIPY TR cadaverine (BC), which binds to native LPS strongly, specifically recognizing lipid A, and is competitively displaced by compounds displaying an affinity for lipid A. The assay clearly discriminates differential affinities between polymyxin B and its nonapeptide derivative, which could not be accomplished using the dansyl-cadaverine displacement method [48]. As will be reported in Part II, quantitative effective displacement (ED_{50}) values can be determined rapidly in a microtiter plate format using single-wavelength detection of emission intensity, rather than full

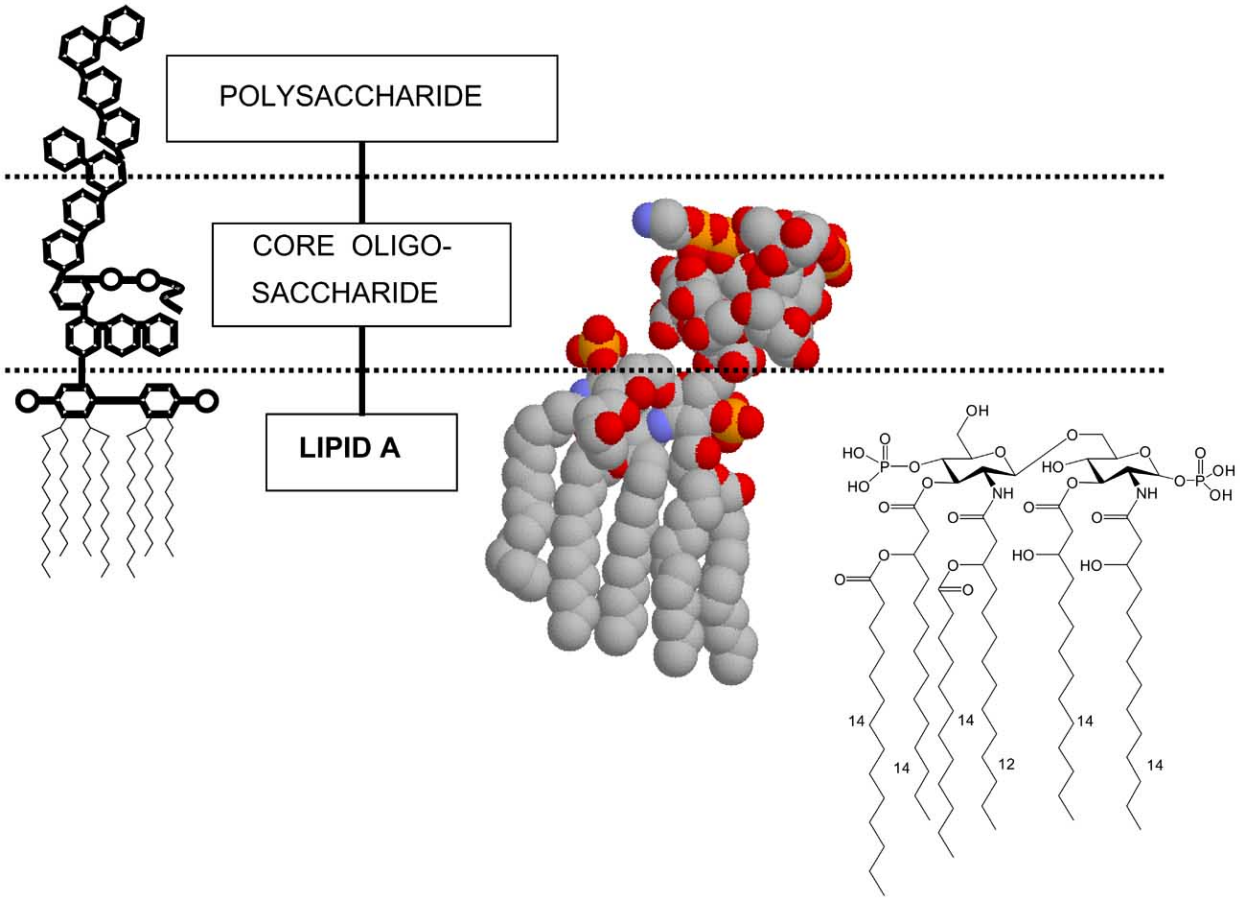

Fig. (2). Schematic (left), crystal structure (middle) of lipopolysaccharide (LPS), and the structure of Lipid A. The polysaccharide domain is disordered in the crystal structure [12].

emission scans. The spectral properties of the BODIPY fluorophore are ideally suited for screening diverse structural classes of compounds, including those with conjugated aromatic groups, or with chromophores in the 260-500 nm range. The fluorescent probe: LPS complex is stable under physiologically relevant salt concentrations, resulting in the rapid rejection of spurious binders interacting *via* non-specific ionic interactions, and, therefore, in greatly improved dispersion of ED_{50} values.

MATERIALS AND METHODS

BODIPY TR cadaverine (5-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl) phenoxy) acetyl) amino) pentylamine, hydrochloride, BC) was purchased from Molecular Probes (Eugene, OR, USA). LPS from *E. coli* serotype 0111:B4 and lipid A from *E. coli*, serotype K12, D31m4 were purchased from List Biological Labs, Inc., CA, USA. All other chemicals and reagents were from Sigma Chemicals (St. Louis, MO).

Fluorescence Experiments

Fluorescence measurements were performed on a Fluoromax-3 (Jobin Yvon Inc., NJ, USA) at 25ºC, using a continuously stirred cuvette. The BC excitation wavelength was 580 nm. Corrected emission spectra were recorded in the photon-counting mode with both emission and excitation monochromators bandpasses at 1 nm. To analyze binding stoichiometries, fluorometric titrations were carried out in Tris buffer (pH 7.4, 50 mM) by the addition of aliquots of concentrated solutions of either LPS (1 mg/ml) or lipid A (5 mg/ml), to avoid dilution effects, into a solution of BC (5 μM). Both the LPS and lipid A solutions were vortexed and sonicated extensively to obtain clear monodisperse solutions. To study the effects of ionic strength of solvent on the stability of the BC:LPS/lipid A complex, aliquots of saline (4 M) were successively added to a mixture of lipid A (2.5 μM) and either BC (10 μM) or DC (10 μM) in water. Steady-state emission anisotropy and polarization were recorded at 25ºC, with 10 nm/10 nm bandpasses, using automated Glan-Thompson calcite polarizing prisms built into the spectrofluorometer. Anisotropy, r, was computed by the equation: $(I_{VV} - G.I_{VH})/(I_{VV} + 2G.I_{VH})$, where the first and second subscripts refer, respectively, to the excitation and emission polarizer orientation (V: 90º; H:0º). G, the grating factor was measured as I_{HV}/I_{HH} for each experiment.

Fluorescence Displacement Assay

The displacement assay was performed by the addition of small (2-10 μl) aliquots of a solution of either Polymyxin B

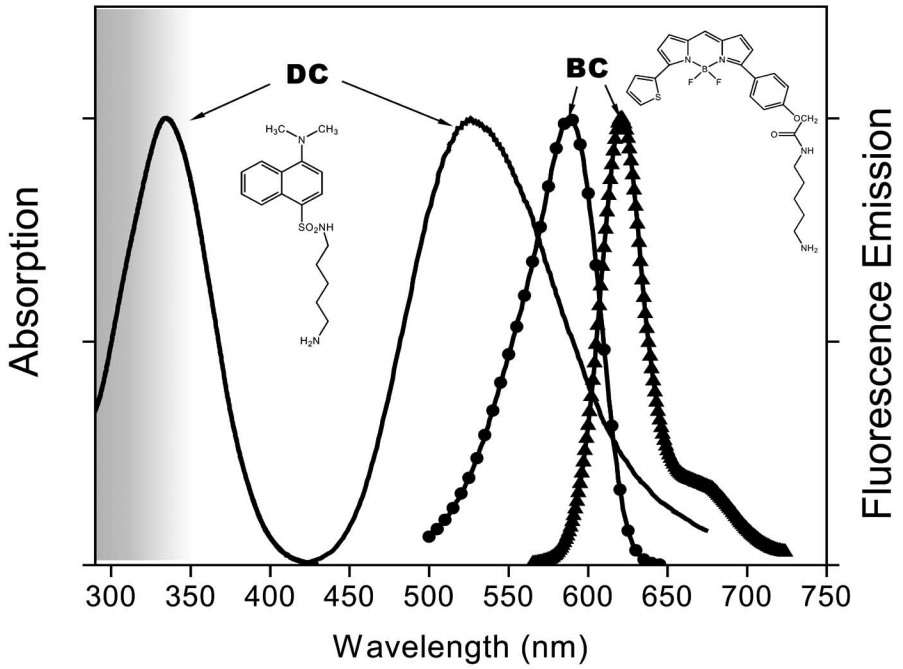

Fig. (3). Structures, excitation and emission spectra of DC and BC in 50 mM Tris buffer, pH 7.4. The excitation and emission λ_{max} for DC and BC are, respectively, 333, 525, and 583 and 620 nm. The shaded gray zone on the left of the graph represents the zone of interference by common conjugated aromatic chromophores in commercially-available focused small molecule libraries.

(PMB) (26.9 μM) or PMB nonapeptide (PMN) (26.7 μM), in 50 mM tris buffer, pH 7.4, to a continuously stirred mixture (2 ml volume) of either LPS (10 μg/ml) or lipid A (10 μM) and BC (10 μM) in a quartz cuvette. PMB is a prototype lipid A binder, and is used as the reference compound [46, 47]. An estimate of probe occupancy (the fraction of BC bound to lipid A bound to BC), was calculated from $(F_0 - F)/(F_0 - F_{max})$, where F_0 is the fluorescence intensity of BC alone, F_{max} is the intensity in the presence of lipid A/LPS at the saturation concentration, and F, the intensities of the lipid A/LPS:BC mixtures at varying displacer concentrations [47].

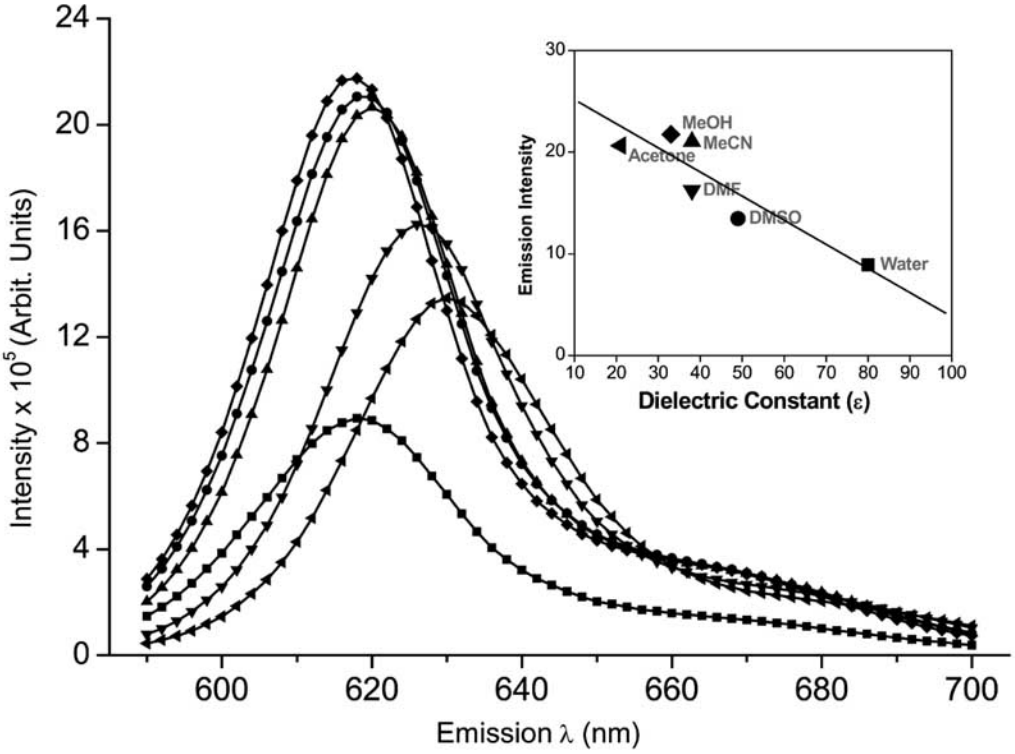

Fig. (4). Emission spectra of BC in an eluotropic series of solvents. Excitation: 580 nm. Bandpasses: 1 nm/1 nm. Anticipated emission intensity enhancements as well as blue-shifts in λ_{max} occur in solvents of low polarity. *Inset*: Correlation of BC emission intensity versus solvent polarity.

Isothermal Calorimetry (ITC)

All ITC experiments were performed using a VP-ITC Microcalorimeter (Microcal Inc., MA, USA). A typical titration experiment involved 35 consecutive injections at 360 s intervals consisting of 3 μl injections of LPS into the sample cell (cell volume: 1.4119 ml) containing the ligand, at 37 °C in Tris buffer (pH 7.4, 50 mM). The titration cell was stirred continuously at 310 rpm. Care was taken to ensure that both LPS and ligand were dissolved in the same buffer, and appropriate control experiments (LPS injected into buffer, BC injected into buffer) were performed. The resulting data were then analyzed using Microcal's ITC data analysis package, VP Viewer 2000, which uses the scientific plotting software, Origin 7 (Origin Lab. Corp., MA, USA).

RESULTS AND DISCUSSION

The excitation and emission spectra of Dansyl-cadaverine (DC) and BODIPY-cadaverine (BC) in 50 mM Tris buffer, pH 7.4, are shown in Fig. (**3**). Unlike DC whose λ_{ex} is 333 nm, the λ_{ex} of 580 nm for BC is far removed from the absorption bands of most chromophores found in commercially available small molecule libraries. Although BC displays the anticipated behavior of enhanced quantum yields accompanied by blue shifts in an eluotropic series of solvents with decreasing polarity (Fig. (**4**)), the titration of LPS against BC results in a concentration-dependent and saturable quenching of BC fluorescence (Fig. (**5**)). While the saturability suggests the formation of a BC:LPS complex, the quenching was unexpected since many fluorescent probes (including DC [47]) manifest in emission intensity enhancements when bound to lipid A, signifying that the fluorophore senses the apolar local environment of the hydrophobic polyacyl domain of lipid A. The stoichiometry of the putative BC:LPS complex cannot be determined precisely since the glycosylation pattern of the polysaccharide domain of LPS is highly variable, and the molecular weight of LPS, consequently, as determined by MALDI-TOF, shows a broad envelope of masses between 2000 and 8,000 a.m.u. (S.A. David, unpublished results). However, assuming a monomeric mass of 5000 a.m.u., the BC: lipid A stoichiometry obtained from the inflection point of the titration curve depicted in Fig. (**5**) corresponds to 1.25:1.

Titration experiments were carried out with purified, structurally homogeneous, *bis*-phosphoryl *E. coli* lipid A in order to verify that BC was binding to the lipid A moiety of LPS (the biologically relevant target portion, and to rule out non-specific adsorption to the polysaccharide domain). This also allowed a more precise determination of binding stoichiometry, and that the binding to lipid A resulted also in fluorescence quenching. As evident from Fig. (**6**), lipid A also induces a concentration-dependent attenuation in the emission intensity of BC, the inflection point of which corresponds to a 1:1 complex, confirmed also by identical stoichiometry values obtained by steady-state anisotropy measurements (Fig. (**6**)). Although we were initially surprised by the unexpected quenching, an examination of the literature yielded several examples of polyamine-bearing fluorophores undergoing quenching when complexed with anions [49-51] due to the formation of intramolecular charge-transfer complexes. We had established that DC binds lipid A primarily *via* salt-bridges between the free protonatable amine group of cadaverine and the glycosidic phosphate group on lipid A [47]. Given that the same functional group is also present on BC, we surmise that the mode of binding of BC to lipid A should be indistinguishable from that of DC, and the formation of ionic H-bonds between the amine and phosphate would be compatible with an intramolecular proton-transfer in the BODIPY fluorophore.

Of additional advantage, particularly for high-throughput screening applications, is the observation that the quenching of BC occurs when complexed with either purified lipid A or native LPS, and is not accompanied by concomitant blue shifts, as was observed with DC [47]. The K_D for DC:lipid A is 17 μM [47], but that of DC:LPS is on the order of 380 μM (unpublished), resulting in poor signal-to-noise ratios, and displacement experiments could be reliably performed only with lipid A. Although lipid A is the target region of LPS, it is preferable to design primary-screen experiments using native LPS, since it is LPS and not lipid A that is clinically relevant. An additional significant advantage with BC is that the quenching can be monitored at a single wavelength (620 nm), unlike DC, whose emission λ_{max} varies from 550 nm (free) to about 470 nm (saturated with lipid A), thus obviating full emission scans at every displacer dilution.

The affinity of DC to lipid A is relatively weak (17 μM), being driven primarily by electrostatic interactions between the protonated primary amine of cadaverine and the anionic lipid A phosphate groups [47]. This poses two problems: (a) DC is easily displaced, resulting in poor discrimination of true ED_{50} values for high-affinity binders; (b) the stability of the DC:lipid A complex is easily perturbed by monovalent cations, necessitating the use of very low, non-physiological (<10 mM) ionic strength conditions. This problem is further compounded by the fact that LPS and lipid A, being amphipathic, self-assemble into lamellar supramolecular aggregates with the polyacyl domain of the lipid in the interior, and the anionic backbone on the solvent-exposed surface [52, 53], presenting an electrostatic "double-layer" at the solvent interface [54-57] to which cations non-specifically absorb [41, 48, 58]. The interfacial electrostatic potential (ψ_0, in mV) is related to the solvent salt concentration by the Gouy equation [41, 54]:

$$\sinh [e\psi_0/(2kT)] = A\sigma/(C)^{0.5}$$

where σ is the charge density at the surface, e, the electronic charge, C, the molar concentration of the univalent cation, k, the Boltzmann constant, and T, the absolute temperature. A is a constant equal to $1/(8N\varepsilon_r\varepsilon_0 kT)$, where N is the Avogadro's number, ε_r is the dielectric constant, ε_0 is the permittivity of free space. The operational equation at 25° C for monovalent ions reduces to the form [57]

$$\psi_0 = 51.4 \sinh^{-1} [135/(AC^{1/2})$$

where C is the molar concentration of the univalent cation, and A is the area in $Å^2$ per unit charge at the interfacial surface. A is computed to be 55 $Å^2$, assuming two charges and a cross-sectional area of 1.10 nm^2 for lipid A [59].

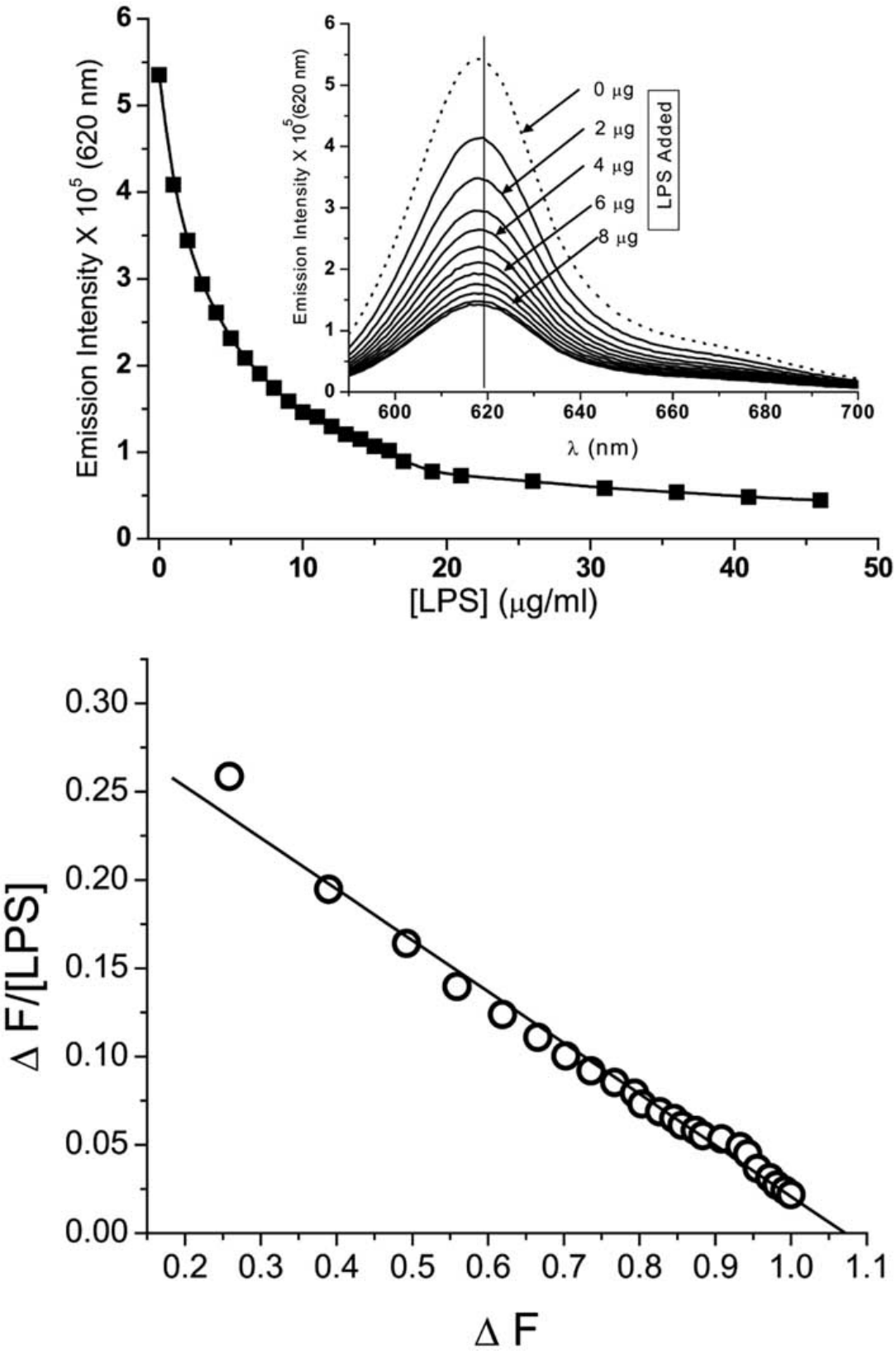

Fig. (5). *Top*: Fluorescence titration of *E. coli* 0111:B4 LPS against 5μM BC in 50mM Tris, pH 7.4. Excitation: 580 nm. Bandpasses: 1 nm/1 nm. The inflection point corresponds to 12 μg LPS added. Assuming a mass of 5000 amu for LPS, the inflection point of the titration curve at 20 μM corresponds to a BC:LPS stoichiometry of 1.25:1. *Inset*: Emission spectra of BC alone in buffer (dotted line). The addition of LPS results in concentration dependent quenching of BC with no changes in λ_{max} (618 nm). Bottom: Scatchard-type plot of the binding of BC to LPS. The computed K_D (-1/slope) is 3.448 μM.

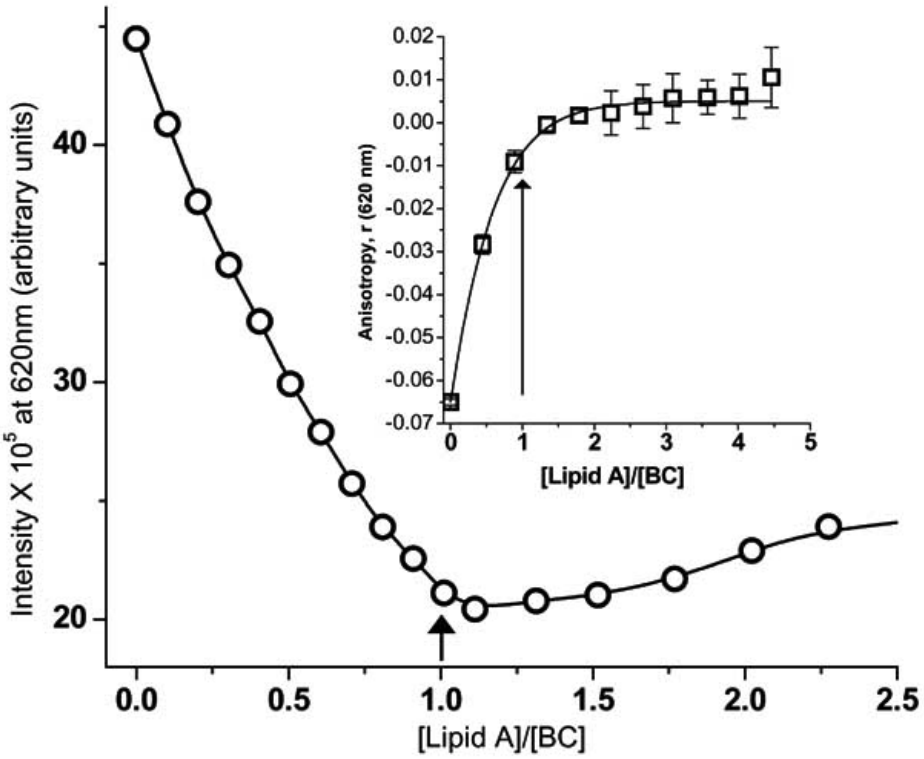

Fig. (6). Fluorescence titration of lipid A from *E. coli*, serotype K12, D31m4 against 5μM BC in 50mM Tris, pH 7.4. Excitation: 580 nm. Bandpasses: 1 nm/1 nm. Fluorescence quenching is plotted against Lipid A/BC molar ratio. Note that the stoichiometry of the complex is 1:1 (arrow). *Inset*: Steady-state emission anisotropy of lipid A/BC titration. Excitation: 580 nm. Emission: 620nm. Bandpasses: 5 nm/5 nm. Note that the inflection point corresponds also to a 1:1 lipid A/BC stoichiometry (arrow). Error bars denote SD computed on four measurements per data-point.

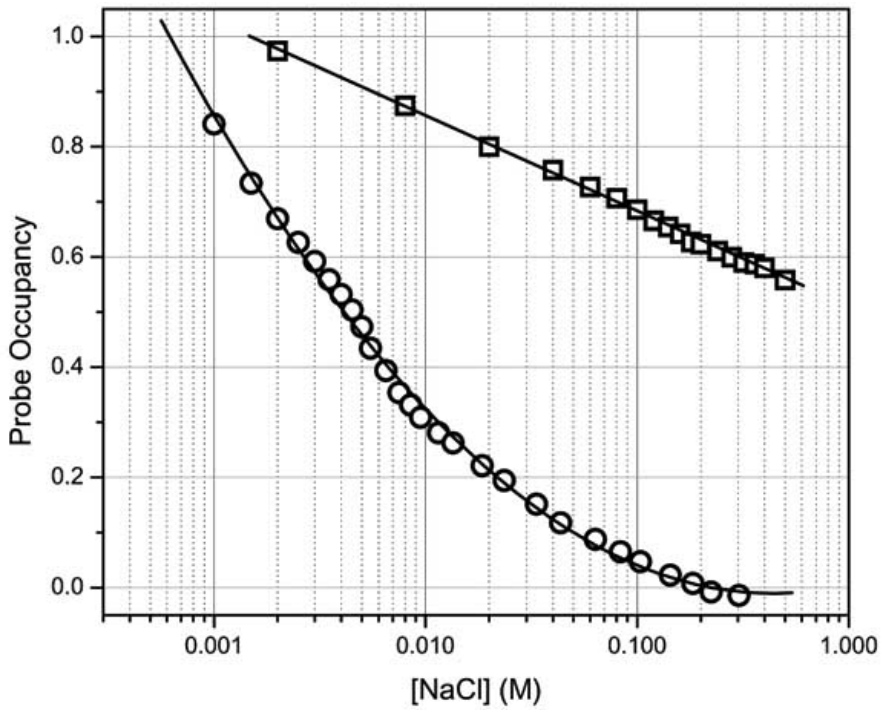

Fig. (7). Effect of solvent ionic strength on the stability of DC:lipid A (open circles) and BC:Lipid A (open squares) complexes. A 1:1 stoichiometry of probe:lipid A (5μM/5μM) was used. 2-5 μl aliquots of 4M NaCl were successively added to the complexes contained in 2.0 ml Milli-Q water. Excitation and emission wavelengths were as noted in Materials and Methods with 1 nm/1 nm bandpasses for BC and 5 nm/5 nm for DC. Fraction of bound probe (occupancy) was computed as described in Materials and Methods. Essentially identical results were obtained in titrations with probe:LPS complexes.

Using the above formalisms, we had computed that ψ_0 for lipid A/LPS is 195 mV in pure water, falling off to 150 mV at 50 mM aqueous NaCl, and to 125 mV at 200 mM NaCl [41]. The attenuation of ψ_0 under suitable ionic strength conditions greatly reduces nonspecific adsorption of polycations, allowing the discrimination of true binders. As shown in Fig. (**7**), at 50 mM NaCl, 50% of bound DC is displaced from lipid A, whereas only ~3% of bound BC is displaced, indicating that the BC:lipid A complex is far more stable, which is consistent with the observed K_D value of 3.448 μM (Fig. (**5**)).

Having characterized in detail the interactions of BC with LPS and lipid A, we analyzed the binding of polymyxin B (PMB), a cyclic decapeptide antibiotic obtained from *Bacillus polymyxus*, and its nonapeptide derivative (PMN). Polymyxin B specifically recognizes and binds to the lipid A portion of LPS [60], and the resultant complex is virtually devoid of toxicity [61, 62]. PMB has served as a "gold standard" for endotoxin-sequestering agents and is routinely used in experimental studies when a biological effect is to be verified as that due to LPS, or to abrogate activity of contaminating LPS. We were keen to compare PMB and PMN, since it is known that PMN is considerably less potent than PMB in its endotoxin-neutralization activity, and yet we were unable to document differences in binding affinity using the DC displacement method [48]. It has been shown using isothermal titration calorimetry that both PMB and PMN bind LPS with an approximately 1:1 stoichiometry (range 1.1 – 1.3, depending on the type of LPS used), and the differences in the interaction with LPS between the two stem primarily from slightly lower enthalpy (ΔH) changes at low peptide:LPS concentrations [63], consistent with K_D values obtained using surface-plasmon resonance methods, of 6.666×10^{-7} M, and 2.645×10^{-6} M, respectively, for PMB and PMN, using *E. coli* 0555:B5 LPS in 10 mM HEPES, pH 7.4, with 250 mM NaCl [64].

The addition of the polymyxins results in de-quenching of LPS-bound BC fluorescence, manifesting in emission intensity enhancements. The results depicted in Fig. (**8**) clearly show the differences in affinity between PMB and

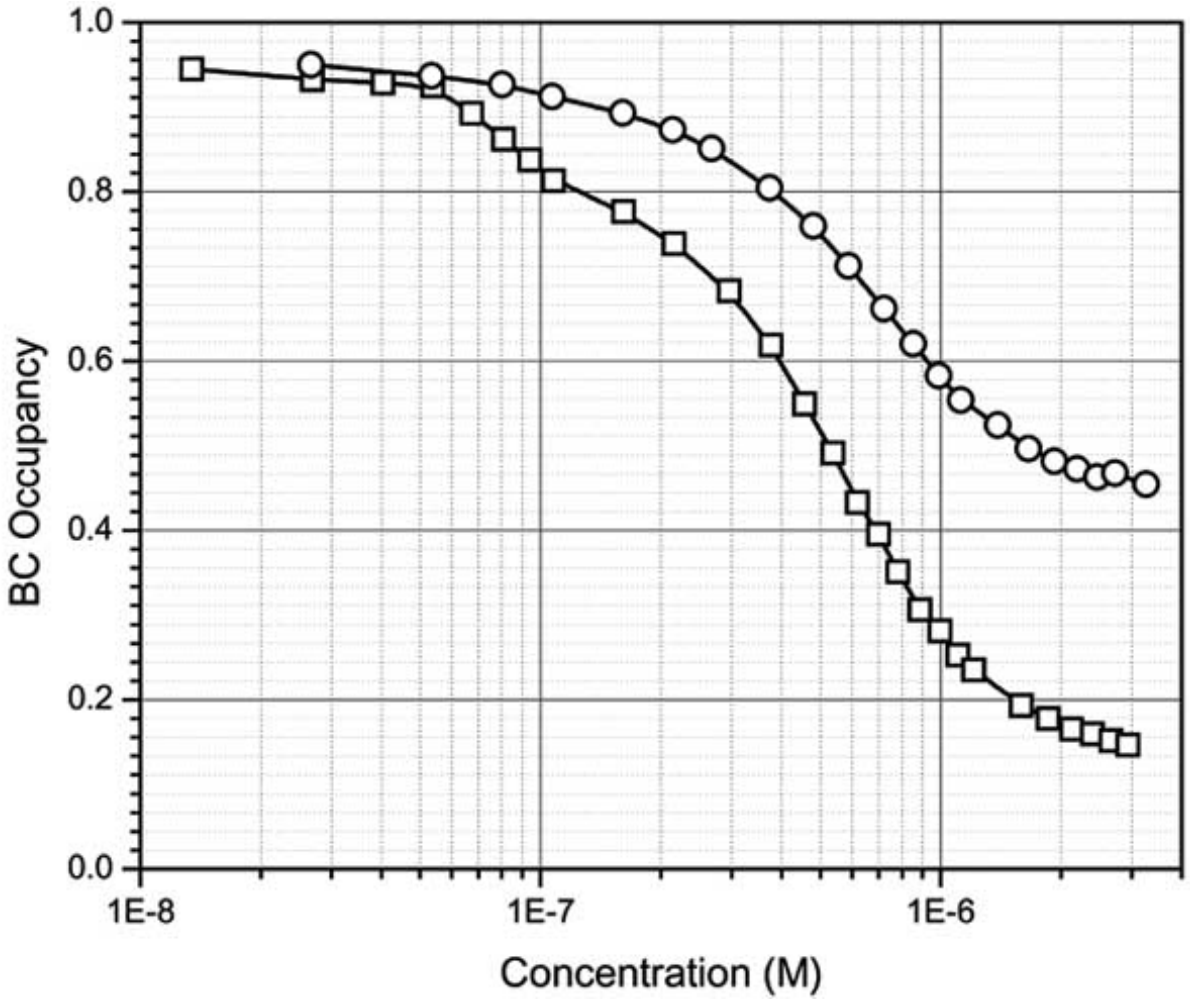

Fig. (8). Displacement of LPS-bound BC by polymyxin B (open squares) and polymyxin B nonapeptide (open circles). The displacement results in de-quenching of BC, manifest in emission intensity enhancements. The fraction of LPS-bound BC (probe occupancy) was computed as described in Materials and Methods. [LPS]: 10 μg/ml; [BC]: 10 μM. Buffer: 50 mM Tris, pH 7.4. 2-5 μl aliquots of PMB/PMN (at 20 μM concentration) were successively added to the cuvette containing the BC:LPS complex. Excitation: 580 nm; Emission: 620 nm. Bandpasses: 1 nm/1 nm.

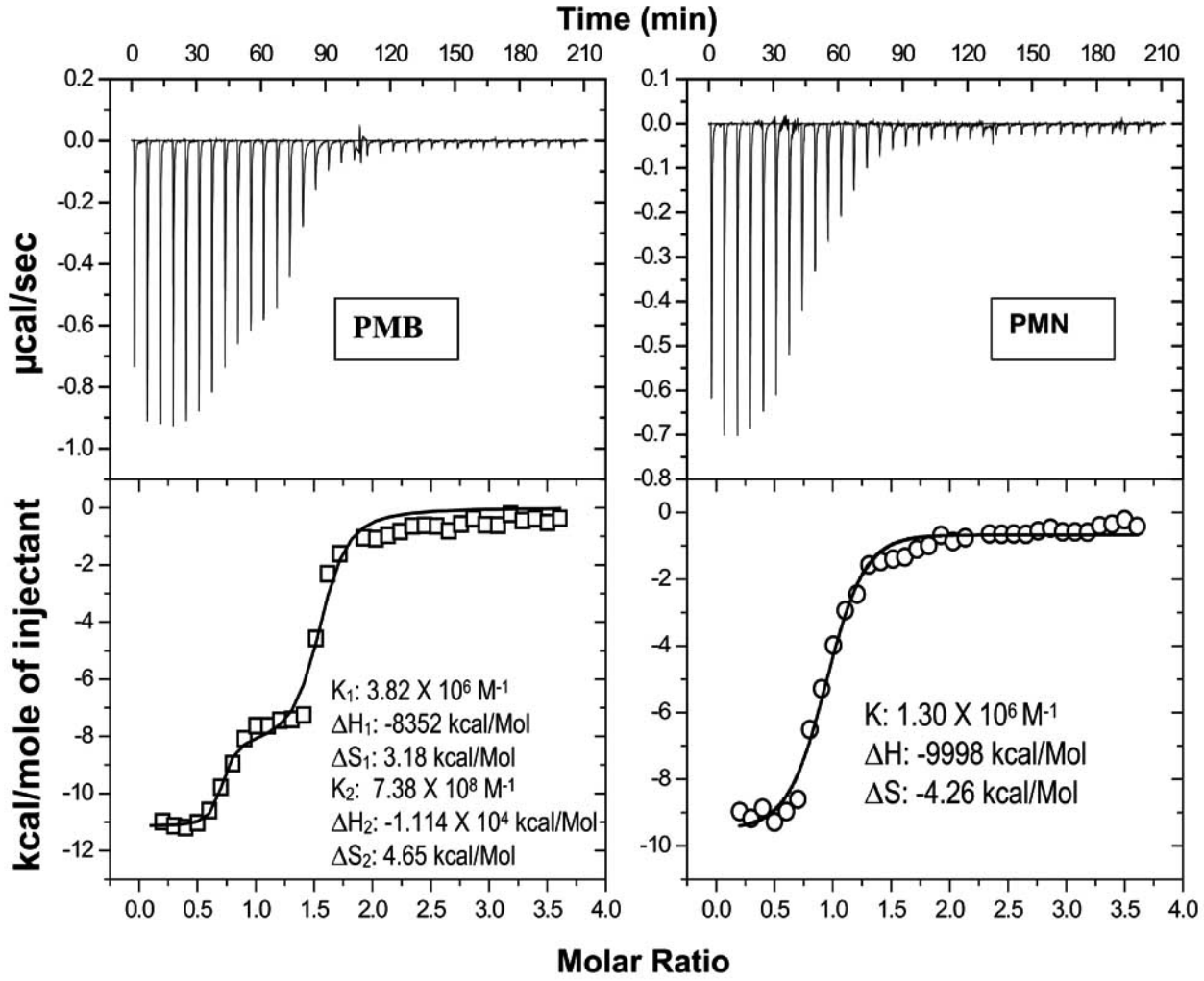

Fig. (9). ITC titrations of LPS with PMB (left panel; open squares) and PMN (right panel; open circles). The binding is exothermic, with a biphasic interaction of PMB to LPS with an apparent 1:1 and 2:1 stoichiometry, in contrast to PMN, which binds LPS monophasically with a 1:1 stoichiometry. The dissociation constants for PMB are: 2.618×10^{-7} and 1.355×10^{-9} M, and for PMN: 7.69×10^{-7} M. ITC experiments were carried out in 50 mM Tris buffer, pH 7.4, at 37ºC. LPS at 5 mg/ml was injected in 3 µl aliquots into the ITC cell containing 15 µM PMB or 20 µM PMN.

PMN toward LPS. In the case of PMB, the displacement curve is distinctly biphasic as has been reported previously by us using DC [64], with the ED_{50} values of the two components corresponding to 7×10^{-8} and 4×10^{-7} M respectively. PMN, in contrast, induces a monophasic displacement of BC (Fig. (**8**)) with an ED_{50} of 2×10^{-6} M. These observations are concordant with thermodynamic parameters obtained from ITC experiments (Fig. (**9**)), showing a biphasic binding isotherm for the PMB:LPS complex, but not for PMN:LPS. As expected, the binding of both PMB and PMN are enthalpically-driven, given that the geometry of the binding is governed primarily by electrostatic interactions, with little change in the overall conformation of the peptide from the free to the LPS-bound state [35]. The dissociation constants obtained from the ITC experiments (Fig. (**9**)) for the two phases of binding of PMB are, 1.355×10^{-9} and 2.618×10^{-7} M, and for PMN, 7.69×10^{-7} M. It is to be noted that the ED_{50} values obtained from BC displacement experiments cannot be numerically compared to the K_Ds derived from ITC experiments since the ED_{50} values are dependent on the ligand (LPS) concentrations as well as the saturation of the probe (BC) [47, 48].

There are significant differences between using dansyl-polymyxin B (originally described by Schindler and Teuber [65], and later used extensively for the characterization of outer membrane permeabilization by polycationic antimicrobials [66-70]) and DC or BC. Both dansyl-polymyxin B and DC bear the dansyl fluorophore which does not have optimal spectral properties as discussed above. In addition, the binding of dansyl-polymyxin B to LPS involves both hydrophobic and ionic interactions and is consequently very strong, requiring very high concentrations of LPS-binding ligands to induce significant displacement of the bound probe; the resultant complex is also insoluble and the particulate precipitates pose considerable problems in accurately quantifying fluorescence intensities. The interaction of fluorescently labeled polyamines (such as cadaverine) is primarily electrostatically driven, and is thus

ideal for screening for scaffolds that interact with the lipid A backbone.

In conclusion, we have characterized a novel fluorescent probe for LPS, and developed a displacement assay method which is able to clearly discriminate relative affinities of binding between PMB and PMN towards native LPS under conditions of physiologically relevant salt concentrations. The spectral properties of the BODIPY fluorophore are superior to that of dansyl [47, 66, 67], allowing rapid and quantitative determination of bound probe concentrations at a single wavelength, and is thus ideally suited for high-throughput screening applications.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

BC = BODIPY TR cadaverine; (5-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl) phenoxy)acetyl)amino)pentyl-amine, hydrochloride, BC)

DC = Monodansylcadaverine

ITC = Isothermal titration calorimetry

LPS = Lipopolysaccharide

PMB = Polymyxin B

REFERENCES

[1] Gelfand, J.A.; Shapiro, L. *New Horizons,* **1993**, *1*, 13.
[2] Gasche, Y.; Pittet, D.; Sutter, P. *Outcome and prognostic factors in bacteremic sepsis. In Clinical trials for treatment of sepsis*, W.J. Sibbald and J.L. Vincent, eds. (Berlin: Springer-Verlag), **1995**, pp. 35-51.
[3] *MMWR,***1990**, *39*, 31.
[4] Martin, G.S.; Mannino, D.M.; Eaton, S.; Moss, M. *N. Engl. J. Med.,***2003**, *348*, 1546.
[5] Cross, A.; Opal, S.M. *J. Endotoxin Res.,***1994**, *1*, 57.
[6] Lüderitz, O.; Galanos, C.; Rietschel, E.T. *Pharmacol. Ther.,***1982**, *15*, 383.
[7] Rietschel, E.T.; Kirikae, T.; Schade, F.U.; Mamat, U.; Schmidt, G.; Loppnow, H.; Ulmer, A.J.; Zähringer, U.; Seydel, U.; Di Padova, F.; *et al. FASEB J.,***1994**, *8*, 217.
[8] Rietschel, E.T.; Brade, L.; Lindner, B.; Zähringer, U. In *Bacterial endotoxic lipopolysaccharides, vol. I. Molecular biochemistry and cellular biology*, D.C. Morrison and J.L. Ryan, eds. (Boca Raton: CRC Press), **1992**, pp. 1-41.
[9] Holst, O.; Ulmer, A.J.; Brade, H.; Rietschel,E.T. In *Immunotherapy of infections*, N. Masihi, ed. (New York, Basel, Hong Kong: Marcel Dekker, Inc.), **1994**, pp. 281-308.
[10] Rietschel, E.T.; Wollenweber, H.W.; Sidorczyk, Z.; Zähringer, U.; Lüderitz, O. In *Bacterial lipopolysaccharides: structure, synthesis and biological activities*, L. Anderson and F.M. Unger, eds. (Washington, D.C.: Am. Chem. Soc. Symp. Ser.), **1983**, pp. 195-212.
[11] Galanos, C.; Lüderitz, O.; Rietschel, E.T.; Westphal, O.; Brade, H.; Brade, L.; Freudenberg, M.A.; Schade, U.F.; Imoto, M.; Yoshimura, S.; Kusumoto, S.; Shiba, T. *Eur. J. Biochem.,***1985**, *148*, 1.
[12] Ferguson, A.D.; Hofmann, E.; Coulton, J.; Diedrichs, K.; Welte, W. *Science,***1998**, 2215.
[13] Kirikae, T.; Schade, F.U.; Zähringer, U.; Kirikae, F.; Brade, H.; Kusumoto, S.; Kusama, T.; Rietschel, E.T. *FEMS Immunol. Med. Microbiol.,***1994**, *8*, 13.
[14] Kusumoto, S.; Inage, M.; Chaki, H.; Imoto, M.; Shimamoto, T.; Shiba, T. In *Bacterial lipopolysaccharides: structure, synthesis, and biological activities*, L. Anderson and F.M. Unger, eds. (American Chemical Society), **1983**, pp. 237-254.
[15] Hoffman, W.D.; Natanson, C. *Anesth. Analg.,***1993**, *77*, 613.
[16] Bone, R.C.; Sprung, C.L.; Sibbald, W.J. *Crit. Care Med.,***1992**, *20*, 724.
[17] Bone, R.C.; Balk, R.A.; Cerra, F.B.; Dellinger, R.P.; Fein, A.M.; Knaus, W.A.; Schein, R.M.; Sibbald, W.J. *Chest,***1992**, *101*, 1644.
[18] Bone, R.C. *Clin. Chest Med.,***1996**, *17*, 175.
[19] Ulevitch, R.J. *Immunol. Res.,***2000**, *21*, 49.
[20] Ulevitch, R.J.Tobias, P. *Curr. Opin. Immunol.,***1999**, *11*, 19.
[21] Dinarello, C.A. *Curr. Top. Microbiol. Immunol.,***1996**, *216*, 133.
[22] Dinarello, C.A. *Adv. Immunol.,* **1989**, *44*, 153.
[23] Dinarello, C.A. *J. Infect. Dis.,* **1991**, *163*, 1177.
[24] Michie, H.R.; Manogue, K.R.; Spriggs, D.R.; Revhaug, A.; O'Dwyer, S.; Dinarello, C.A.; Cerami, A.; Wolff, S.M.; Wilmore, D.W. *N. Engl. J. Med.,* **1988**, *318*, 1481.
[25] Beutler, B.; Poltorak, A. *Drug Metab. Dispos.,* **2001**, *29*, 474.
[26] Ingalls, R.R.; Heine, H.; Lien, E.; Yoshimura, A.; Golenbock, D. *Infect. Dis. Clin. North Am.,* **1999**, *13*, 341,vii.
[27] Lien, E.; Means, T.K.; Heine, H.; Yoshimura, A.; Kusumoto, S.; Fukase, K.; Fenton, M.J.; Oikawa, M.; Qureshi, N.; Monks, B.; Finberg, R.W.; Ingalls, R.R.; Golenbock, D.T. *J. Clin. Invest.,* **2000**, *105*, 497.
[28] Poltorak, A.; He, X.; Smirnova, I.; Liu, M.Y.; Huffel, C.V.; Du, X.; Birdwell, D.; Alejos, E.; Silva, M.; Galanos, C.; Freudenberg, M.; Ricciardi, C.P.; Layton, B.; Beutler, B. *Science,* **1998**, *282*, 2085.
[29] Ulevitch, R.J.Tobias, P.S. *Annu. Rev. Immunol.,* **1995**, *13*, 437.
[30] Ulevitch, R.J. *Adv. Immunol.,* **1993**, *53*, 267.
[31] Marshall, J.C. *Nature Rev. Drug. Disc.,* **2003**, *2*, 391.
[32] Zeni, F.; Freeman, B.; Natanson, C. *Crit. Care Med.,* **1997**, *25*, 1097.
[33] David, S.A.; Balaram, P.; Mathan, V.I. *J. Endotoxin. Res.* ,**1995**, *2*, 99.
[34] David, S.A. In *Endotoxins in health and disease*, H. Brade, S.M. Opal, S.N. Vogel, and D.C. Morrison, eds. (New York: Marcel Dekker), **1999**, pp. 413-422.
[35] Bhattacharjya, S.; David, S.A.; Mathan, V.I.; Balaram, P. *Biopolymers,* **1997**, *41*, 251.
[36] David, S.A.; Bhattacharjya, S.; Mathan, V.I.; Balaram, P. *J. Endotoxin. Res.,* **1994**, *1* (suppl.), A60.
[37] David, S.A.; Balaram, P.; Mathan, V.I. *Med. Microbiol. Lett.,* **1993**, *2*, 42.
[38] David, S.A.; Mathan, V.I.; Balaram, P. *Biochim. Biophys. Acta,* **1992**, *1123*, 269.
[39] David, S.A.; Awasthi, S.K.; Balaram, P. *J. Endotoxin Res.,* **2000**, *6*, 249.
[40] David, S.A.; Bechtel, B.; Annaiah, C.; Mathan, V.I.; Balaram, P. *Biochim. Biophys. Acta,* **1994**, *1212*, 167.
[41] David, S.A.; Mathan, V.I.; Balaram, P. *J. Endotoxin. Res.,* **1995**, *2*, 325.
[42] Vaara, M. *FEMS Microbiol. Lett.,* **1983**, *18*, 117.
[43] David, S.A.; Awasthi, S.K.; Wiese, A.; Ulmer, A.J.; Lindner, B.; Brandenburg, K.; Seydel, U.; Rietschel, E.T.; Sonesson, A.; Balaram, P. *J. Endotoxin Res.,* **1996**, *3*, 369.
[44] David, S.A.; Perez, L.; Infante, M.R. *Bioorg. Med. Chem. Lett.,* **2002**, *12*, 357.
[45] David, S.A.; Silverstein, R.; Amura, C.R.; Kielian, T.; Morrison, D.C. *Antimicrob. Agents Chemother.,* **1999**, *43*, 912.
[46] David, S.A. *J. Molec. Recognition,* **2001**, *14*, 370.
[47] David, S.A.; Balasubramanian, K.A.; Mathan, V.I.; Balaram, P. *Biochim. Biophys. Acta,* **1992**, *1165*, 147.
[48] David, S. A. Analysis of the desirable structural properties in potential endotoxin antagonists. **1995**, *Ph.D. Thesis*. Christian Medical College Hospital, Vellore, India.
[49] Ref Type: Video Recording
[50] Huston, M.E.; Akkaya, E.U.; Czarnik, A.W. *J. Am. Chem. Soc.,* **1989**, *111*, 8735.
[51] Huston, M.E.; Engleman, C.; Czarnik, A.W. *J. Am. Chem. Soc.,* **1990**, *112*, 7054.

[52] Akkaya, E.U.; Huston, M.E.; Czarnik, A.W. *J. Am. Chem. Soc.,* **1990**, *112*, 3590.
[53] Brandenburg, K.; Seydel, U. *Biochim. Biophys. Acta,* **1984**, *775*, 225.
[54] Seydel, U.; Labischinski, H.; Kastowsky, M.; Brandenburg, K. *Immunobiol.,* **1993**, *187*, 191.
[55] Eisenberg, M.; Gresalfi, T.; Riccio, T.; McLaughlin, S. *Biochemistry,* **1979**, *18*, 5213.
[56] Winiski, A.P.; McLaughlin, A.; McDaniel, R.V.; Eisenberg, M.; McLaughlin, S. *Biochemistry,* **1986**, *25*, 8206.
[57] Winiski, A.P.; Eisenberg, M.; Langner, M.; McLaughlin, S. *Biochemistry,* **1988**, *27*, 386.
[58] Usui, S. Electrical double layer. Surfactant Science Series. Kitahara, A. and Watanabe, A. **1984**, New York, Marcel Dekker. Electrical Phenomena at interfaces. Fundamentals, measurements and applications.
[59] Ref Type: Serial (Book,Monograph).
[60] Kim, J.; Mosior, M.; Chung, L.A.; Wu, H.; McLaughlin, S. *Biophys. J.,* **1991**, *60*, 135.
[61] Seydel, U.; Eberstein, W.; Schröder, G.; Brandenburg, K. *Z. Naturforsch.,* **1992**, *47c*, 757.
[62] Morrison, D.C.; Jacobs, D.M. *Immunochemistry,* **1976**, *13*, 813.
[63] Stokes, D.C.; Shenep, J.L.; Fishman, M.L.; Hidner, W.K.; Bysani, G.K.; Rufus, K. *J. Infect. Dis.,* **1989**, *160*, 52.
[64] Stokes, D.C.; Shenep, J.L.; Fishman, M.; Hilder, W.K.; Bysani, G.K.; Rufus, K. *J. Infect. Dis.,* **1989**, *160*, 52.
[65] Brandenburg, K.; Moriyon, I.; Arraiza, M.D.; Lewark-Yvetot, G.; Koch, M.H.J.; Seydel, U. *Thermochim. Acta,* **2002**, *382*, 189.
[66] Thomas, C.J.; Surolia, A. *FEBS Lett.,* **1999**, *445*, 420.
[67] Schindler, P.R.; Teuber, M. *Antimicrobial Agents and Chemotherapy,* **1975**, *8*, 95.
[68] Moore, R.A.; Bates, N.C.; Hancock, R.E. *Antimicrob. Agents Chemother.,* **1986**, *29*, 496.
[69] Peterson, A.; Hancock, R.E.W.; McGroarty, E.J. *J. Bacteriol.,* **1985**, *164*, 1256.
[70] Piers, K.L.; Brown, M.H.; Hancock, R.E.W. *Antimicrob. Agents. Chemother.,* **1994**, *38*, 2311.
[71] Scott, M.G.; Vreugdenhil, A.C.; Buurman, W.A.; Hancock, R.E.; Gold, M.R. *J. Immunol.,* **2000**, *164*, 549.
[72] Wu, M.; Maier, E.; Benz, R.; Hancock, R.E. *Biochemistry,* **1999**, *38*, 7235.

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