Anion Receptors

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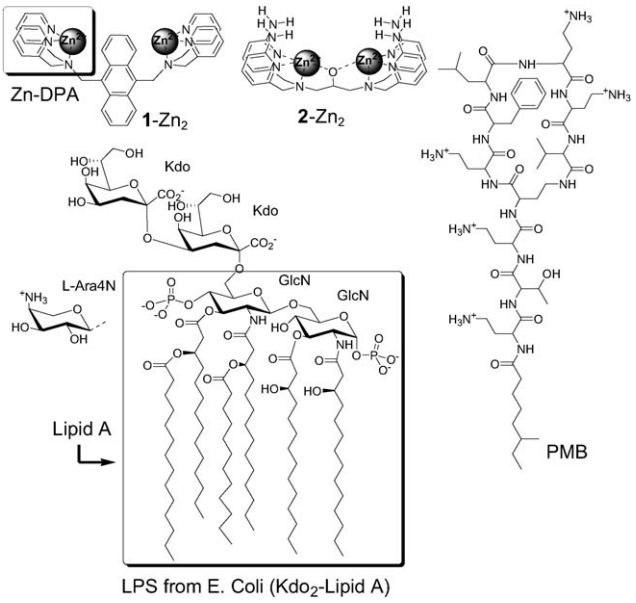
Effective Binding and Sensing of Lipopolysaccharide: Combining Complementary Pattern Recognition Receptors**

*V. Ganesh, Karin Bodewits, S. Josefin Bartholdson, Daniela Natale, Dominic J. Campopiano, and Juan C. Mareque-Rivas**

The development of molecules that bind and detect anions is of considerable importance in many areas of chemistry and biology.[1] Despite major research activity and progress in this area, the creation of synthetic hosts that bind anions with high affinity in water (pH ≈ 7) remains extremely challenging. This task is even more complicated if binding and detection has to occur in clinically relevant solutions, because these solutions contain many chemical species which can compete with the target anion for the receptor. Arguably the most common strategy to create effective synthetic anion receptors is to use organic molecules with convergent H-bond donors, but in general, the anion is bound with high affinity ($K_a = 10^4$–$10^7\,M^{-1}$) only in organic media.[1] Using this strategy, Sessler and co-workers have shown that a single NH···Cl interaction can increase the binding affinity of a calix[4]pyrrole chloride receptor from 10^5 to $10^7\,M^{-1}$ in acetonitrile.[2] Several groups have recently opted for coordination complexes to preorganize H-bonding groups[3a] or form strong metal–anion interactions in water.[3b–f,4] However, examples of synthetic receptors that, for a given biologically important anion, are as effective as those occurring naturally remain elusive: nature creates extremely effective receptors using multiple H-bonding contacts with the anion.[1,5]

An important anion target is lipopolysaccharide (LPS). LPS is the main component of the outer membrane of Gram-negative bacteria and is a potent stimulant of the mammalian immune response.[6,7] At low concentrations, LPS has beneficial biological effects, but at higher levels it can cause an excessive immune response leading to septic shock (self-poisoning of the body), organ failure, and ultimately death. Septic shock is the most common cause of death in intensive care units, and its treatment costs $17 billion annually in the US alone.[8] The primary immunostimulatory component of LPS is the lipid A core (also known as endotoxin; Scheme 1). LPS varies among different bacterial genus, species, and serotypes, but for most LPSs, two 2-keto-3-deoxyoctonate (kdo) units are linked to lipid A, each carrying negative charge in the carboxylate group at neutral pH. These sugars, combined with the 1 and 4′ phosphate moieties on the lipid A, make LPS a highly negatively-charged molecule. As the glucosamine disaccharide core is decorated with various long-chain fatty acid esters and amides, LPS has amphipathic properties.[9]



Scheme 1. Structures of dinuclear zinc(II) complexes, polymyxin B (PMB), and LPS from *E. coli*, along with the L-Ara4N modification, which attaches to the 4′ phosphate group.

Several natural and synthetic peptides bind LPS,[10] and most of them exploit electrostatic and H-bonding interactions. The prototype is polymyxin B (PMB; Scheme 1), for which the K_d of the PMB-LPS complex has been estimated to be 1 μM.[11a] The glycoprotein CD14 is a LPS receptor in mammalian immune cells and forms a CD14-LPS complex with a K_d of 1 μM.[11b] The strongest LPS receptor is the serum glycoprotein LPS binding protein (LBP), which binds the lipid A region of LPS with a K_d of approximately 1 nM.[11c] Given the extreme toxicity of LPS, it is very important to develop methods to detect it, which requires not only a suitable receptor but also an effective signal transduction method. A lipid-functionalized polydiacetylene liposome colorimetric sensor and a fluorescence sensor consisting of a CD14-derived LPS-binding peptide terminally labeled with organic fluorophores have recently been reported.[12] Mammalian immune cells detect LPS using a complex machinery

[*] Dr. V. Ganesh, K. Bodewits, S. J. Bartholdson, D. Natale, Dr. D. J. Campopiano, Dr. J. C. Mareque-Rivas
School of Chemistry, University of Edinburgh
Edinburgh EH9 3JJ (UK)
Fax: (+44) 131-650-4743
E-mail: juan.mareque@ed.ac.uk

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of LPS pattern recognition receptors, and it is believed that a potassium ion channel is involved in the electrochemical signaling process.[13]

Herein, we report a dinuclear zinc(II) complex with convergent hydrogen bonding features, **2**-Zn_2, which binds LPS two orders of magnitude more strongly than either the natural LPS binding agent PMB or **1**-Zn_2 (Scheme 1). The complex **2**-Zn_2 binds LPS in neutral water with very high affinity ($K_a = 1.3 \times 10^7\,M^{-1}$), which is only matched by the LPS binding protein. Moreover, **2**-Zn_2 coupled to electrochemical impedance spectroscopy (EIS) as a mode of signal transduction provides a simple and new technique to effectively detect and distinguish LPS from different bacterial species.

We immobilized endotoxic *E. coli* LPS (kdo_2-lipid A) on self-assembled monolayers (SAMs) of decanethiol (DT) onto gold surfaces by exploiting hydrophobic interactions. This was achieved by dipping the SAM-coated surface into a 40 nM–40 μM solution of LPS. LPS immobilization was initially confirmed by X-ray photoelectron spectroscopy (see Supporting Information). Binding of LPS was remarkably effective, presumably because several hydrophobic chains can interact with the DT monolayer. Thus, the DT monolayer acts as effective pattern-recognition receptor for the hydrophobic part of LPS.

Binding events were then probed by measuring the electron transfer (ET) blocking behavior of the SAMs to redox-active species in solution using EIS.[14] The charge-transfer resistance R_{CT} was determined by fitting the experimental data to a semicircle and determining its intercept on the x axis based on the modified Randle's equivalent circuit.[15] The DT-modified electrode has a R_{CT} value of $(5.90 \pm 1.09) \times 10^5\,\Omega\,cm^2$ compared to $9.30\,\Omega\,cm^2$ for the bare gold electrode, meaning that the monolayer film of DT effectively blocks the ET to the solution-based redox probe $[Fe(CN)_6]^{3-/4-}$. Upon addition of LPS (40 nM–40 μM), R_{CT} sharply increases, reaching a maximum of $(1.48 \pm 0.25) \times 10^6\,\Omega\,cm^2$ (Figure 1 and Supporting Information). The increase in R_{CT} is probably related to both an increase in negative charge and thickness of the molecular monolayer. As other amphipathic molecules are able to bind DT and cause a similar electrochemical response (Figure 1), DT and this signal alone cannot be used for LPS detection purposes. For this we need to mimic the sensing apparatus of our immune system and use another strong LPS binding agent to recognize and bind its hydrophilic part.

PMB is a natural LPS binding agent that interacts with the carboxylate and 4′ phosphate moieties,[11a] whereas **1**-Zn_2 binds dianionic phosphate derivatives, such as phosphorylated peptides, with high affinity in water ($K_a = 10^4$–$10^5\,M^{-1}$).[3d] Moreover, Smith et al. discovered that complexes with zinc(II) dipicolylamine units (Zn-DPA, Scheme 1) have potent antimicrobial activity, and that by linking these units to organic dyes it is possible to create useful fluorescence imaging probes for bacterial cells due to their affinity for anionic phospholipids.[3b,4] We have been developing metal complexes with H-bonding cavities to significantly enhance their properties.[16] In this context we developed **2**-Zn_2.[17] We envisage that **2**-Zn_2 binds phosphate esters in a fashion that allows both zinc(II) ions and four amino H-bonding groups to be used for binding to the lipid A fragment of LPS. We modeled this interaction using the recent X-ray structure of the lipid IVa: human MD-2 receptor complex[5d] and the structure of **2**-Zn_2[17a] (Figure 2).

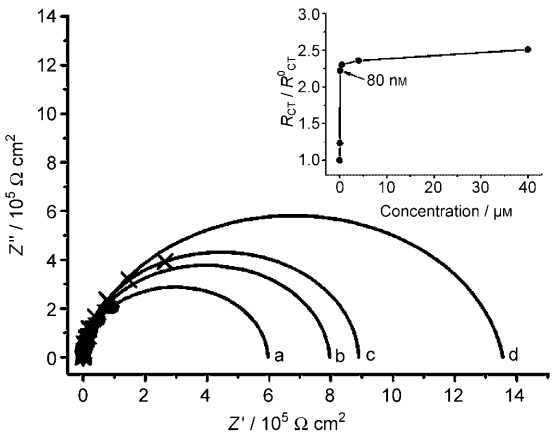

Figure 1. Impedance plots in an aqueous solution containing 1 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M NaCl for the SAM of DT on gold before (a) and after the addition of b) 40 μM stearic acid, c) 40 μM phosphatidylcholine, and d) 80 nM kdo_2-lipid A. The lines show the fitting of the data points using Randle's equivalent circuit.[15] Inset: plot of the ratio R_{CT}/R°_{CT}, (where R°_{CT} is the resistance to the electron transfer reaction before LPS addition) vs. concentration of added kdo_2-lipid A.

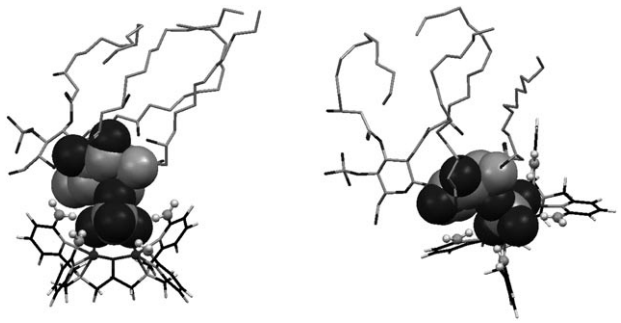

Figure 2. Model of the **2**-Zn_2: lipid IVa interaction. The two views show that the receptor cavity (bottom of both views) is ideal for LPS substrates and can simultaneously coordinate to two zinc(II) ions and hydrogen-bonding to four amino groups for phosphate binding. The glucosamine and 1-phosphate are shown as a space-filling model (center) and the hydrogen atoms on lipid IVa (top) have been omitted for clarity.

The Nyquist impedance plots for the LPS-coated gold electrodes before and after the addition of 100 μM PMB, **1**-Zn_2 and **2**-Zn_2 are shown in Figure 3. Addition of 100 μM PMB, **1**-Zn_2 and **2**-Zn_2 decreases R_{CT} to $(6.75 \pm 1.47) \times 10^5\,\Omega\,cm^2$, $(1.02 \pm 0.1) \times 10^6\,\Omega\,cm^2$ and $(7.59 \pm 1.85) \times 10^5\,\Omega\,cm^2$, respectively. In principle, a decrease in R_{CT} could be due to loss of LPS; however, XPS studies showed that this is not the case. The most likely reason for this decrease in R_{CT} is that binding of these cationic molecules shields to a different extent the negative charge of LPS facilitating the approach of the anionic redox probe. The effect of different concentrations of

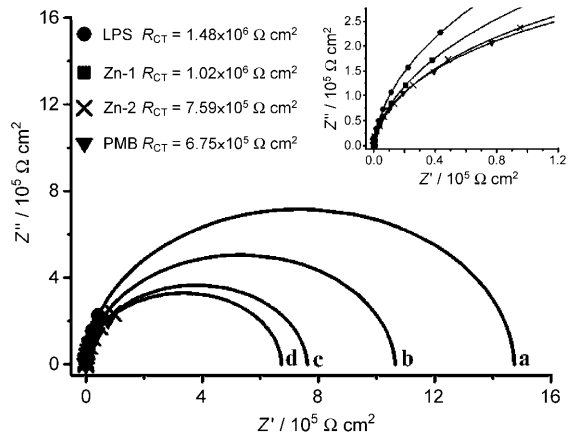

Figure 3. Impedance plots in 1 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M NaCl at the DT monolayer-modified gold electrode containing kdo_2-lipid A before (a) and after the addition of 100 μM b) **1**-Zn_2, c) **2**-Zn_2, and d) PMB.

PMB, **1**-Zn_2, and **2**-Zn_2 was investigated. For PMB and **1**-Zn_2, R_{CT} decreases from 0.1 μM (Figure 4). The binding constants calculated from the changes in R_{CT} using a Langmuir isotherm

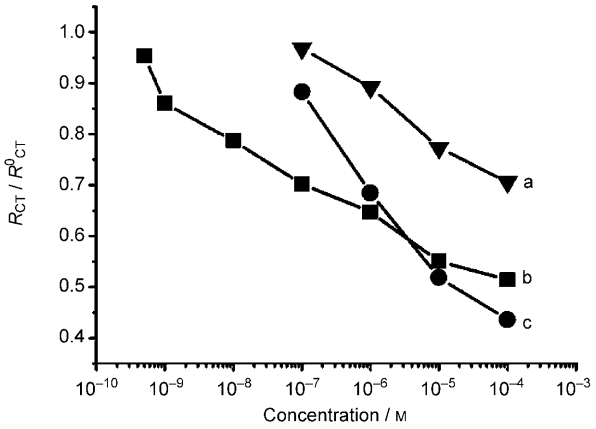

Figure 4. A plot of the ratio of R_{CT}/R^o_{CT} (R^o_{CT} is the charge transfer resistance before the addition of zinc complex or polypeptide) vs. concentration for the addition of a) **1**-Zn_2, b) **2**-Zn_2, and c) PMB to kdo_2-lipid A attached to DT monolayer-modified gold surfaces.

are $1.3\times10^5\,M^{-1}$ for **1**-Zn_2 and $3.3\times10^5\,M^{-1}$ for PMB. These values are in good agreement with previously determined dissociation constants for the PMB-LPS complex[11a] and with the K_d reported by Hamachi et al. for **1**-Zn_2 bound to other phosphorylated species in water solutions.[3d] Remarkably, R_{CT} decreases with as little as 0.5 nM **2**-Zn_2, and the LPS binding constant is $1.3\times10^7\,M^{-1}$. Thus, the hydrogen-bonding cavity increases the LPS affinity of a very good receptor such as **1**-Zn_2 by two orders of magnitude. It is worth noting that CD14, which is a LPS receptor in mammalian cells, also binds LPS two orders of magnitude less strongly than **2**-Zn_2.[11b] The affinity of **2**-Zn_2 for LPS is matched only by LBP.[11c] As a control, we examined whether **2**-Zn_2 causes a decrease of R_{CT} in DT surfaces containing stearic acid, which was the case only at concentrations of 1 μM or higher. Moreover, the change is very small (< 10%) even at a concentration of 100 μM (see Supporting Information). This result suggests that LPS binding occurs by the phosphate groups. Because **2**-Zn_2 binds more strongly to LPS than to any other species that may be anchored to the DT-functionalized electrode (see below), it can be used to detect LPS.

We analyzed the performance of the sensor as a function of kdo_2-lipid A concentration, and found that it performs well with LPS concentrations of 80 nM. For comparison, the recently reported lipid-functionalized polydiacetylene liposomes were used to detect LPS at concentrations of around 100 μM,[12a] whereas the fluorescence sensor based upon the natural LPS binding peptide CD14 was able to detect LPS with a detection limit of 0.5 μM.[12b] To investigate whether this LPS sensing strategy works using solutions containing clinically relevant complex mixtures, we investigated the response to 25–50% fetal calf serum solutions in the absence (see Supporting Information) and presence of LPS. The results obtained show that DT + **2**-Zn_2 also detect LPS in this complex biological mixture (Figure 5). By combining two LPS

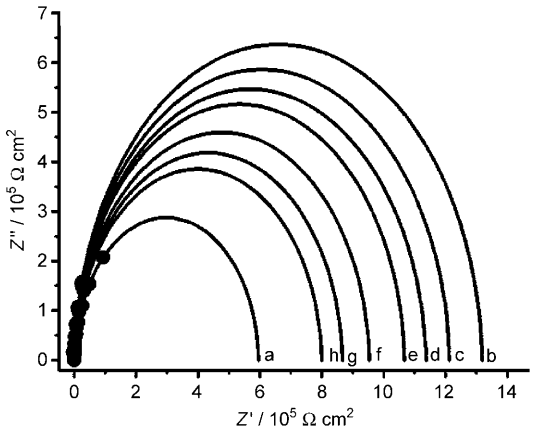

Figure 5. Impedance plots in an aqueous solution containing 1 mM $[Fe(CN)_6]^{3-/4-}$ with 0.1 M NaCl for the SAM of DT (a), SAM of DT + 50% fetal calf serum (FCS) with 1 μM kdo_2-lipid A onto gold electrodes before (b) and after the addition of **2**-Zn_2 1 nM (c), 10 nM (d), 100 nM (e), 1 μM (f), 10 μM (g), and 100 μM (h).

receptors (DT and **2**-Zn_2) this sensor avoids competition and false-positive readings. To compete with LPS and induce false signaling, a compound would have to be as hydrophobic as LPS to bind to the DT receptor and carry a very high negative charge and phosphate ester groups to bind as strongly as LPS does to **2**-Zn_2. This hypothesis was confirmed by investigating the response of this sensor to phosphatidylcholine (40 μM), which is the predominant phospholipid in the membrane of higher eukaryotic cells. Phosphatidylcholine binds the DT-modified electrode, but the change in R_{CT} is much smaller than for 80 nM kdo_2-lipid A (Figure 1). Moreover, addition of **2**-Zn_2 causes a decrease of R_{CT} in DT surfaces containing this phospholipid only if the concentration of the metal complex is 1 μM or higher. In contrast, when the DT electrode was dipped into a solution containing 4 μM kdo_2-lipid A and 40 μM phosphatidylcholine, the increase in R_{CT} is much greater, and addition of 1 nM **2**-Zn_2 already causes a decrease of R_{CT}

(see Supporting Information). Thus, the use of multiple receptors with complementary recognition patterns could become a powerful strategy to develop more effective sensors for other complex biological molecules in biological environments.

Finally, since the presence of the phosphate ester dianionic moieties is critical for binding **2**-Zn_2 to LPS, and the number of these moieties varies among LPS of different bacterial pathogens, we tested whether this zinc(II) complex can distinguish LPS from different bacteria. A good candidate to test this idea is the cystic fibrosis pathogen *Burkholderia cenocepacia*.[18,19] In *Burkholderia* species, LPS is constitutively modified by 4-amino-4-deoxy-L-arabinose (L-Ara4N) on the 4′ position of the lipid A core (see Scheme 1 and Supporting Information), leading to resistance to PMB and cationic antimicrobial peptides.[18–20] For the electrode with kdo_2-lipid A of *E. coli*, R_{CT} decreases by 13% in the presence of 1 nM **2**-Zn_2, but in the case of the L-Ara4N-modified LPS from *B. cenocepacia* strain SAL1, a concentration of 1 μM **2**-Zn_2 is required (see Supporting Information). We suggest this is due to the weaker **2**-Zn_2:LPS binding caused by the L-Ara4N modification of the lipid A core at the phosphate ester groups. The L-Ara4N group is positively charged at pH 7, which neutralizes the negative charge of the lipid A 4′-phosphate group. As a consequence, **2**-Zn_2 is able to distinguish the LPS from *E. coli* from that of *B. cenocepacia*.

In conclusion, our results demonstrate that the benefits of tighter phosphate ester binding to dinuclear Zn-DPA complexes with convergent amino H-bonding groups can be extended to LPS binding and sensing. This approach compares favorably with any natural and synthetic peptide in terms of binding affinity, and with currently available LPS sensing technology in terms of simplicity and efficiency. As in the mammalian innate immune system, this LPS sensing method employs a combination of effective and complementary LPS pattern recognition receptors (DT + **2**-Zn_2 is the most effective) and interactions (hydrophobic, electrostatic, and H-bonding), and electrochemical signaling. The results offer a promising new strategy to create extremely effective anion receptors and sensors that work in water. We are developing similar complexes for improved electrochemical LPS and bacterial detection and to extend their applications to optical sensing, septic shock treatment, and LPS removal.

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