A calixarene-based fluorescence indicator displacement assay for the selective detection of bacteria

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Antimicrobial resistance (AMR) represents a critical global threat in the 21st century [1]. Traditional antibiotics exert significant selective pressure, driving the emergence of resistance even against last-resort treatments. This crisis underscores the urgent need for rapid diagnostic tools capable of discriminating between bacterial classes (Gram-positive, Gram-negative, and Mycobacteria) to ensure timely and targeted therapy [2]. Zwitterionic calixarenes already showed surprising selectivity for Gram-negative bacteria [3]. In this study, we present a novel fluorescence indicator displacement assay (IDA) based on cationic calixarenes for the selective detection of bacterial classes. We synthesized and characterized a series of calix[n]arene derivatives (n = 4, 6, 8) functionalized with pyridinium groups at the upper rim. These derivatives effectively quench the fluorescence of a polyanionic polymer, PPP (poly(2,5-bis(3-sulfonatopropoxy)-1,4-phenylene)), through direct molecular interaction. The binding mechanisms were elucidated using STD (Saturation Transfer Difference) NMR spectroscopy.
The sensing potential was evaluated by monitoring the restoration of PPP fluorescence upon the addition of bacteria, which competitively displace the calixarene ligands from the polymer. On-cell STD NMR analysis of representative bacterial strains provided mechanistic insights into the binding modes. Notably, the calix[4]arene derivative exhibited superior selectivity for Gram-negative bacteria detection [4].
This cost-effective and rapid chemical system represents a significant addition to the diagnostic toolkit in the ongoing fight against AMR.

Acknowledgments:

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References:

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[2] Rajapaksha P, Elbourne A, Gangadoo S, Brown R, Cozzolino D, Chapman J. A review of methods for the detection of pathogenic microorganisms. Analyst 2019; 144(2): 396–411
[3] Rispoli F, Moretti L, Vezzoni CA, Tosi E, Molteni L, Ciaramelli C, et al. Clustering Zwitterionic Amino Acids at the Upper Rim of Cone Calix[4]arene Triggers the Selective Recognition of Gram-Negative Bacterial Envelope. Small Structures. 2025 Jun;6(6):2400547. doi:10.1002/sstr.202400547
[4] Manuscript in preparation.

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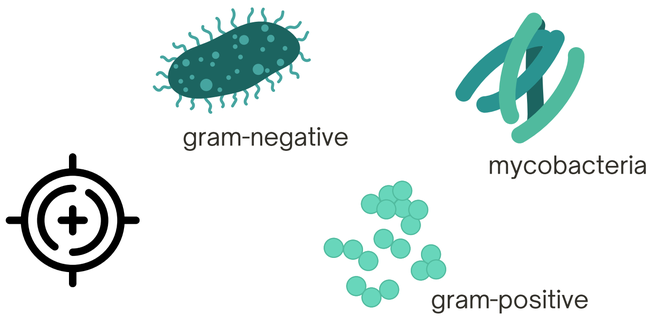
The escalation of **antimicrobial resistance (AMR)** is a critical public health and economic challenge of the 21st century. Driven by the **selective pressure of indiscriminate antibiotic use**, multi-drug resistant (MDR) strains are rapidly disseminating.

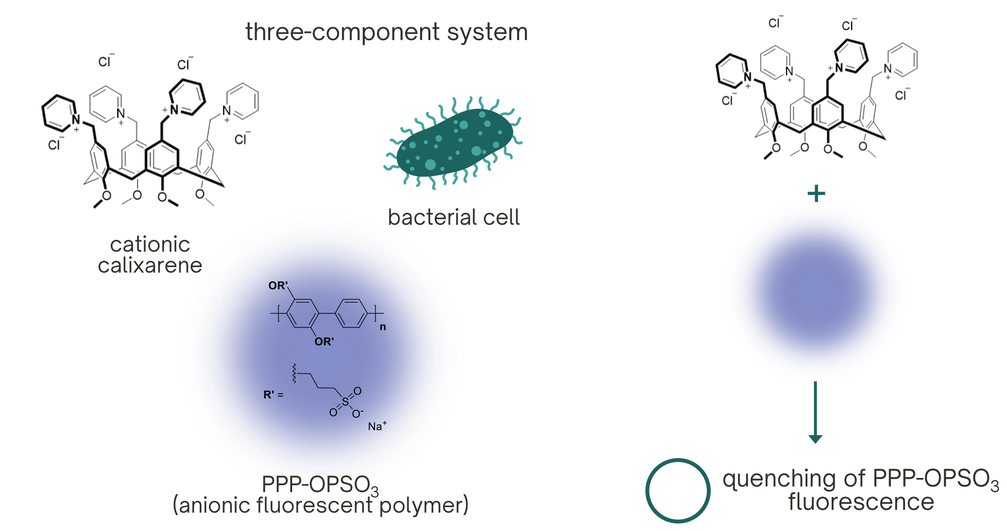
The **diagnostic workflows** still relies on **culture-based methods**

requiring s**everal days to identify pathogens**



There is an **unmet clinical demand** for **rapid tools capable of early pathogen stratification into broad taxonomic classes** to guide targeted therapies.

Here, we describe the development of an **Indicator Displacement Assay (IDA)** based on **cationic calixarenes** designed and synthesised for the selective detection and discrimination of bacterial classes.



1. Structural design and synthesis of the calixarene library

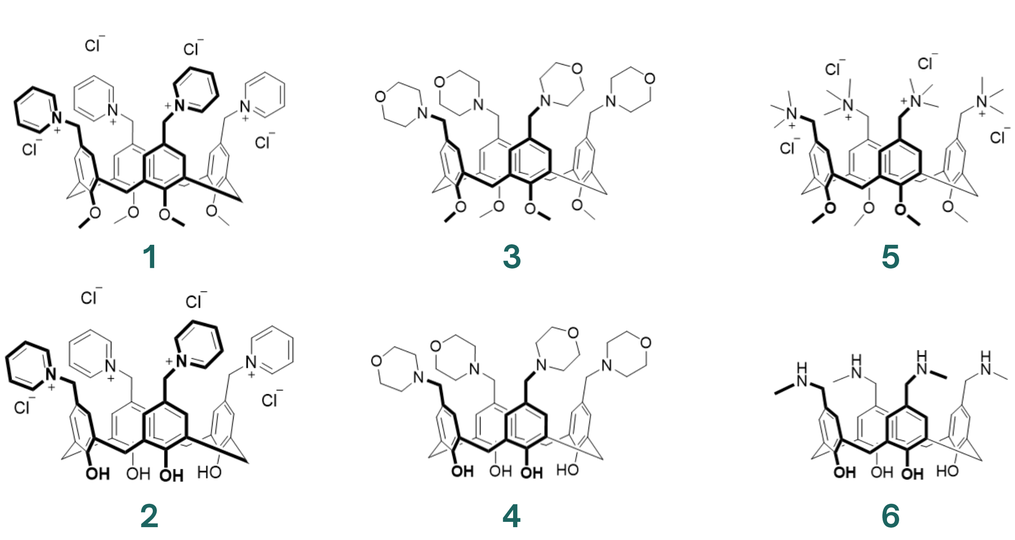


Figure 1. Compounds **1-8** designed, synthesised and tested in this study.

2. Screening of calixarenes for PPP quenching

Pyridinium-functionalized calixarenes display an ability to **quench PPP-OPSO$_3$ fluorescence in the low micromolar range**.

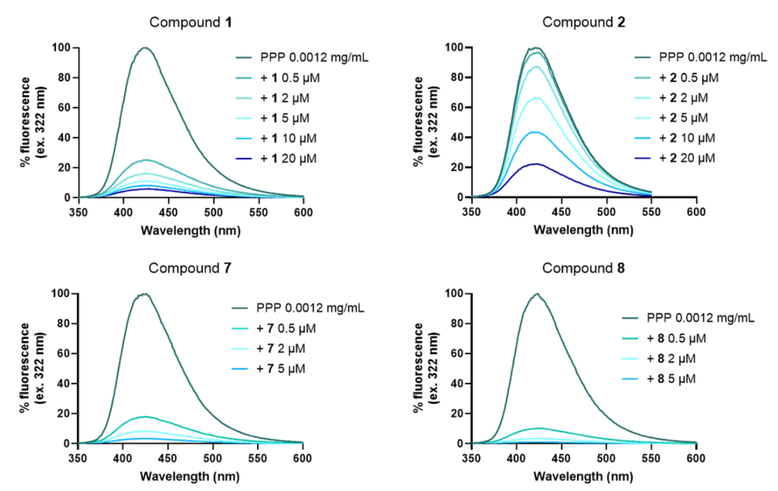


Figure 2. PPP-OPSO$_3$ residual fluorescence after quenching by compounds **1-8** at different concentrations.

3. Assessment of the indicator displacement assay (IDA) potential via bacterial screening

Compound **1** showed an evident, targeted **"turn-on" response** exclusively in the presence of **Gram-negative strains**.

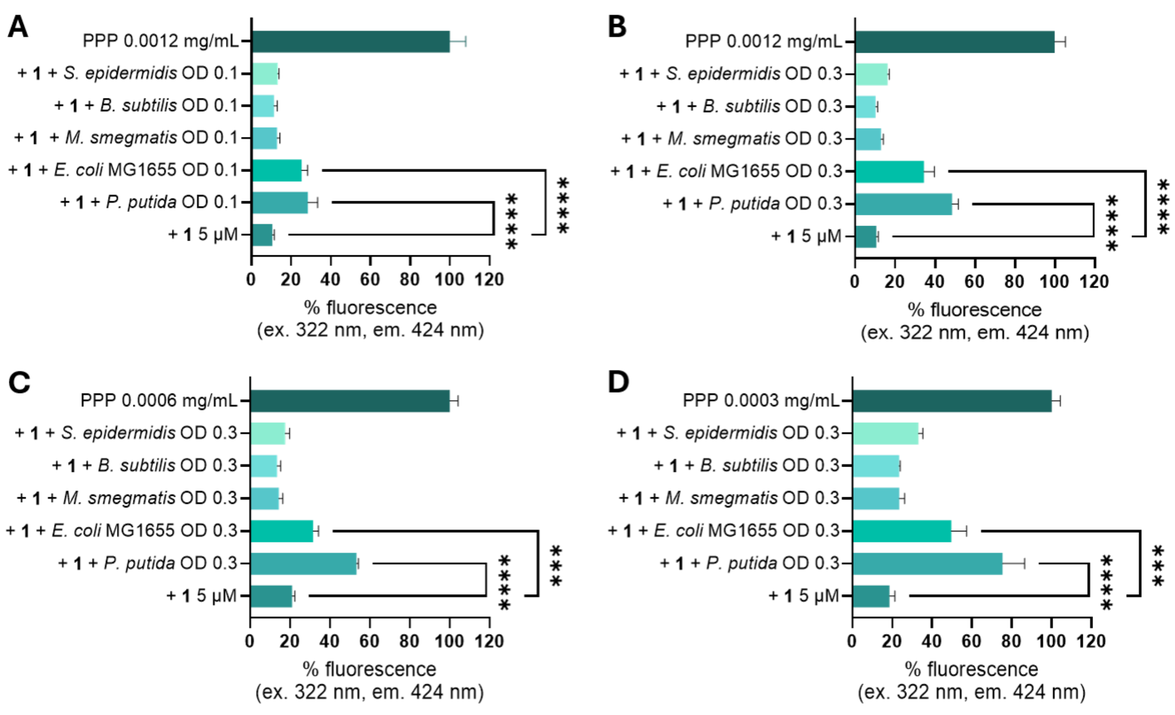


Figure 3. Competitive screening for the assessment of indicator displacement assay (IDA) potential for **1** in the presence of five model bacterial strains at cell optical density (OD_{600}) of 0.1 (A) or 0.3 (B, C and D) and PPP-OPSO$_3$ concentrations of 0.0012 mg/mL (A and B), 0.0006 mg/mL (C), or 0.0003 mg/mL (D). Fluorescence data (λ excitation 322 nm, λ emission 424 nm) are expressed as percentage of PPP-OPSO$_3$ fluorescence intensity, **** P < 0.0001, *** P < 0.0005 vs 1 5 µM + PPP-OPSO$_3$, one-way ANOVA and Dunnett *post hoc* test.

4. Structural confirmation of bacterial binding via on-cell STDD NMR

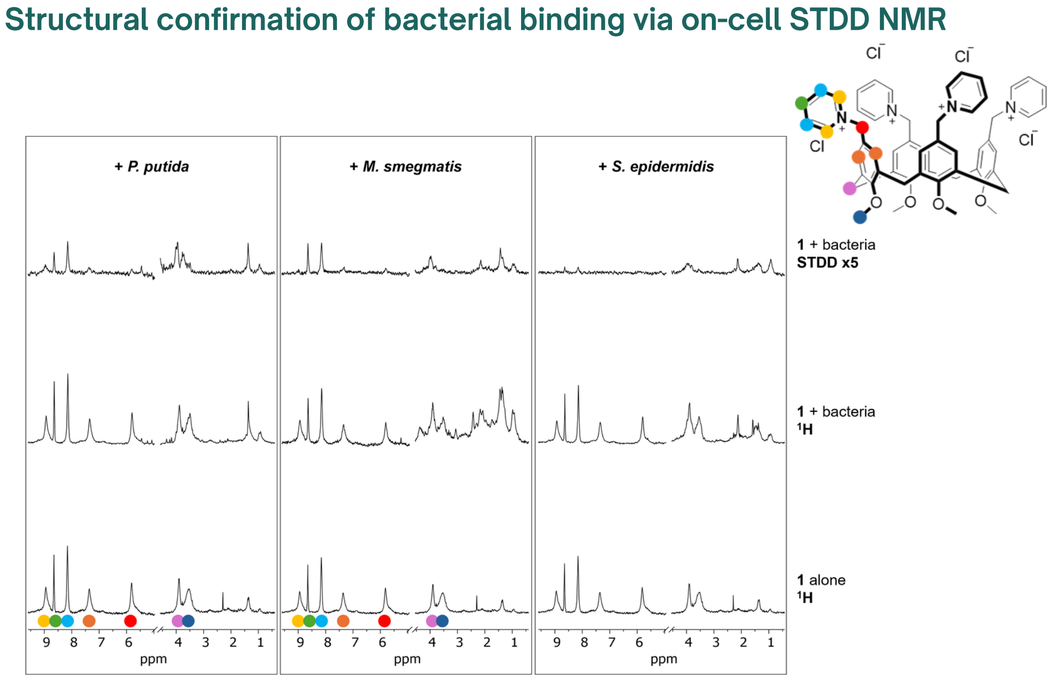


Figure 4. STDD NMR characterization of the interaction between **1** (2 mM) and three different bacterial strains (from left to right, *P. putida*, *M. smegmatis*, *S. epidermidis*, OD_{600} = 1.0) in dPBS, pH 7.4. From bottom: ^{1}H-NMR spectra of **1** alone and in presence of bacterial cells, 1D STDD NMR spectra of **1** in presence of bacterial cells (x5 increment of intensity). ^{1}H NMR spectra were acquired with 80 scans; STD NMR spectra were acquired with 256 scans and 3 s of saturation time, on-resonance frequency of -1.0 ppm for *P. putida* and *M. smegmatis* and of 0.5 ppm for *S. epidermidis*, off-resonance frequency of 30 ppm. All spectra were recorded at 37 °C at 600 MHz.

5. Rationalization of PPP-OPSO$_3$ binding and proposed sensing mechanism

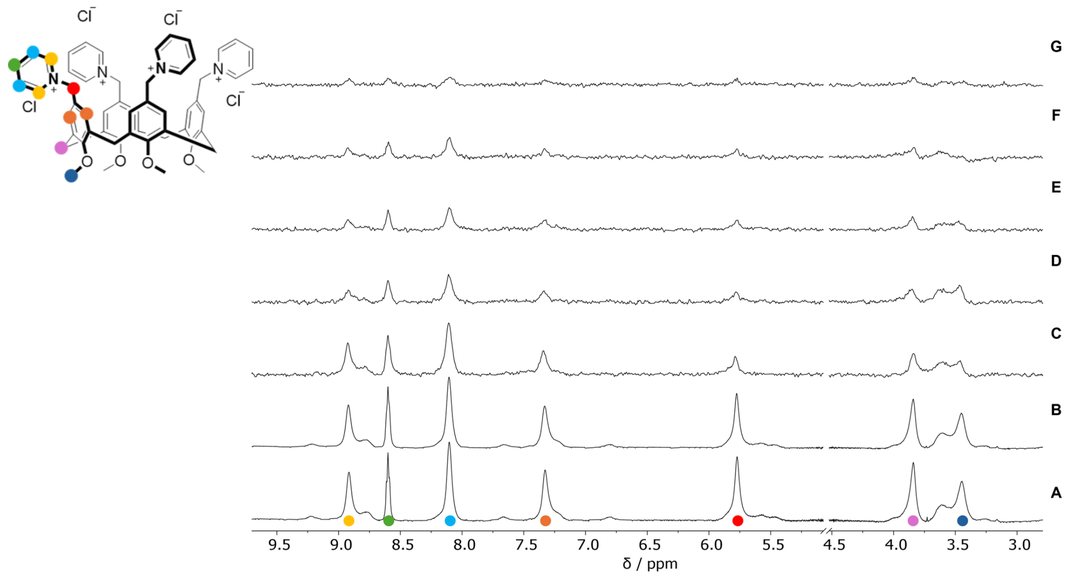


Figure 5. STDD NMR characterization of the interaction between **1** and PPP-OPSO$_3$. (A) ^{1}H NMR spectra of **1** (2mM) alone. (B) ^{1}H NMR spectra of **1** (2 mM) in the presence of PPP-OPSO$_3$ (10 µM). (C-G) STDD NMR spectra of **1** (2mM) in the presence of PPP-OPSO$_3$ (10 µM) with saturation time of 3 s (C), 2 s (D), 1.5 s (E), 1 s (F), 0.65 s (G). ^{1}H NMR spectra were acquired with 80 scans; STD NMR spectra were acquired with 256 scans, on-resonance frequency of 1.65 ppm, off-resonance frequency of 30 ppm. All spectra were recorded in dPBS, pH 7.4, at 37 °C and 600 MHz.

Conclusion

We have successfully developed an **innovative, supramolecular fluorescence IDA** capable of selectively **detecting Gram-negative bacteria in aqueous media**. By combining rational structural design and synthesis, photophysical screenings, and cutting-edge state-of-the-art NMR techniques, this work clarifies the subtle structural parameters that govern non-covalent host-pathogen recognition at the mesoscale.

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3. Rispoli F, Moretti L, Vezzoni C A, Tosi E, Molteni L, Ciaramelli C, Marchiò L, Volpi S, Baldini L, Sansone F, Palmioli A, Airoldi C, Casnati A. Clustering Zwitterionic Amino Acids at the Upper Rim of Cone Calix[4]Arene Triggers the Selective Recognition of Gram-Negative Bacterial Envelope. Small Structures 2025, 6 (6), 2400547.

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