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Modulation of *P. aeruginosa* quorum sensing and host immune response with biomimetic cyclodextrin enzyme models

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Scientists working in the field of biomimetic nanomaterials are usually inspired by evolutionary strategies. Bacteria evaluate their own population density and disease causing processes through a unique chemical communication. Language used for this communication, quorum sensing (QS), has been determined to be *N*-acyl-homoserine lactones (AHLs) for most of the bacteria. AHL hydrolysis by the lactonase enzymes (cysteine hydrolases or metalloenzymes) of host species is known to inhibit QS. Here, we present the first biomimetic quorum quenching approach with the use of supramolecular enzyme mimetics. Thiol bearing (**CD-SH**) and metal chelated (**CD–Cu(II)**) cyclodextrin derivatives are shown to modulate bacterial quorum sensing, and **CD-SH** interferes with the inflammatory immune response of *P. aeruginosa*-infected host human lung cells. Like natural defensive enzymes against bacteria, CD-based enzyme mimetics with a hydrophobic binding pocket reduce pyocyanin production in *P. aeruginosa*, modulate QS, and reduce biofilm formation. The viability of infected lung cancer cells is significantly restored, and mucin and interleukin expressions are reduced upon treatment with **CD-SH**. Considering the role of cytokines and mucin glycoproteins in cystic fibrosis pathogenesis, bacterial attachment and accumulation, the host immune modulatory effect of artificial enzyme mimetics can pave the way for the development of potential new treatment modality for patients suffering from CF.

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Introduction

Rapid emergence of antibiotic resistant bacteria, cost of developing new antibiotics and potential selection of resistant species by the use of any bactericides have driven scientists to seek alternative antibacterial methods.[1] Bacteria are known to evaluate the population density around them through a unique form of chemical communication.[2] Once enough bacteria occupy the local environment, a threshold communication signal is reached, leading to the activation of genes mediating toxin production and biofilm formation. Chemical language used for quorum sensing (QS) has been determined to be *N*-acyl-homoserine lactones (AHLs) for most of the bacteria.[3] Inhibition of AHL production/reception is shown to attenuate pathogenicity.[4] In certain organisms, AHL hydrolysis by lactonase enzymes is used as a defence strategy and is known to act as a quorum quencher.[5] These metalloenzymes catalyse AHL hydrolysis and hence delay the reaching of the threshold AHL concentration required for quorum activation. In this work, we present the first use of artificial metalloenzymes, like natural lactonases, that can degrade AHL and modulate QS. The cyclodextrin artificial enzyme bears hydrophobic cavities that bind AHL through supramolecular interactions. A metal catalyst is embedded into the structure with the intention of catalyzing AHL hydrolysis and inhibiting QS. Knowing that lactonases belonging to the cysteine hydrolase enzyme group can also degrade AHLs, we also investigated quorum quenching and the host immune modulation ability of a thiol bearing cyclodextrin.[6]

CD derivatives studied in this research are intended to be a lactonase mimetic. Lactonases are used by some organisms as an anti-pathogenic defence mechanism. Like the natural enzymes, CD enzymes mimicking the active site are expected to catalyse the hydrolysis of the AHL signals or sequester AHL (Fig. 1). As a result, the QS cascade cannot activate disease causing processes such as exotoxin production. Structures of the cyclodextrin derivatives are shown in Fig. 2 and Fig. S1. Molecules are made of a β-cyclodextrin-based receptor module (yellow container) which binds to AHL through hydrophobic supramolecular interactions. β-CD is a natural cyclic oligosaccharide which has been already

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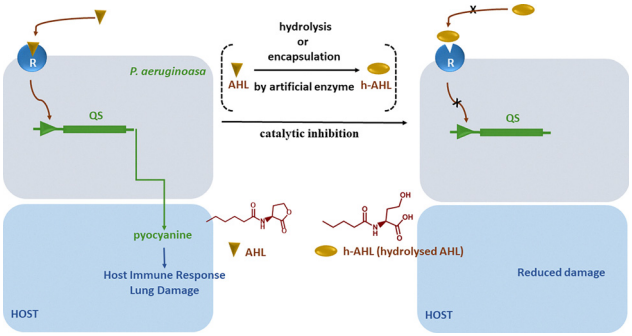

Fig. 1 Proposed method uses small molecules to inhibit QS through hydrolysis and/or supramolecular encapsulation of AHLs. AHL sequestering or generation of hydrolysed AHL (h-AHL) is proposed to modulate QS gene expression and host immune response.

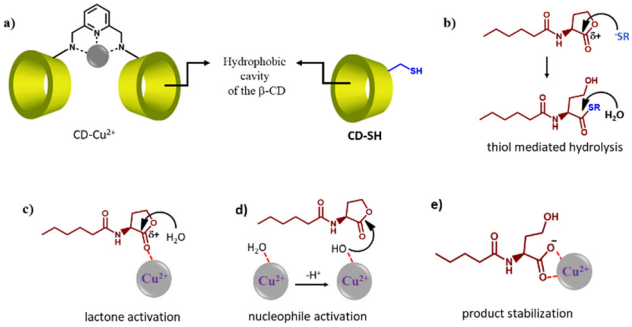

Fig. 2 Schematic representation of CD derivatives (a) and proposed mechanism of AHL hydrolysis by **CD-SH** (b) and by Cu–Cu(II) (c)–(e).

used for drug delivery and food applications.[6] It has a hydrophobic interior that binds hydrophobic guest molecules having alkyl or aromatic units, in aqueous solutions. Encapsulation of natural autoinducers, natural AHLs and aryl substituted synthetic AHLs by the cyclodextrin derivatives has been previously reported.[7] In some of these studies, encapsulation dependent hinderance of AHLs influences QS at high doses. In this work, β-CD is used as a binding pocket of an artificial enzyme scaffold and the structure is further functionalized with 2,6-dimethylamino pyridine to enable coordination to a divalent metal, close to the core CD receptor module (grey sphere, Cu^{2+}, Fig. 2). The use of this metal unit is inspired by natural enzymes such as hydrolase, metalloprotease, nuclease, and lactonase. These enzymes use metal ions in their active site for the catalysis of the hydrolysis reaction.[8] With the same or similar architecture, artificial enzymes made up of small molecules have also been developed to accelerate the rate of ester and phosphate hydrolysis.[9] To date, AHL hydrolysis by the artificial enzyme models and their use as quorum quenchers have not been explored. The role of the metal in the aforementioned enzymes and artificial catalysts is basically to catalyse the reaction using one or a combination of three possible ways, as shown in Fig. 2c–e: the metal can facilitate the reaction by coordinating to the oxygen of an ester (it will be a cyclic ester, lactone in the case of AHL) and further polarizing the C–O bond, making the carbonyl more prone to nucleophilic attack (c); the metal also coordinates to water and leads to ionization at ambient pH in the vicinity of the AHL (d); lastly, the metal can stabilise the carboxylate product and by doing so drive the reaction forward (e).[10]

In biology, Zn^{2+}, Cu^{2+}, and Mn^{2+} divalent metals are the most common ones taking part in the active site of hydrolytic enzymes. In the research, Cu^{2+} is used to catalyse the lactone (AHL) hydrolysis. Since AHL is expected to be brought close to the metal by binding to a cavity, a further rate increase is expected. Previously, β-CD derivatives are used as binding pockets for the substrates of artificial enzymes.[11] Likewise, divalent metals bound to CD are shown to catalyse the hydrolysis of activated esters or phosphates and β-CD itself can catalyse polymerization of lactones through transesterification.[12] Since certain lactonases are cysteine hydrolases, we have used thiol bearing **CD-SH** to hydrolyse AHL through nucleophilic attack by thiolate (Fig. 2b). The thioester intermediate is expected to be hydrolysed.

Experimental

Preparation of CD and CD–Cu(II) and model DAP–Cu(II)

6-Mercapto-6-deoxy-beta-cyclodextrin (**CD-SH**) was obtained commercially (ABCR, AB528256) and used without further purification. CD and **CD–Cu(II)** molecules were synthesised using the experimental procedure described in the literature by stirring 6-monoamino-β-cyclodextrin (0.3 mmol, 351 mg), 2,6-bis(dimethylamino)pyridine (0.1 mmol, 27 mg) and caesium carbonate (0.3 mmol, 38 mg) in dimethyl formamide at room temperature for 24 h.[12] The resulting compound was purified by dialysis (benzoylated dialysis tubing, Sigma-Aldrich, D7884). **CD–Cu(II)** was prepared by stirring CD (100 mg) with copper(II) perchlorate hexahydrate in 0.5 mL of H_2O and 0.5 mL of ethanol for 5 min followed by precipitation of the blue product by pouring the mixture into 20 mL of ethanol.

The model compound (DAP–Cu(II)) which has Cu(II) in its structure but lacks CD was synthesized to understand the effect of Cu(II) without a binding module. 2,6-Diacetyl pyridine (DAP, 100 mg, 0.61 mmol) and copper(II) perchlorate (227 mg, 0.61 mmol) were dissolved in 1 mL of dH_2O and 1 mL of ethanol and the mixture was stirred for 5 min at RT. Solvent was evaporated in a reduced vacuum to yield the DAP–Cu(II) model compound with quantitative yield. ^{1}H NMR (d-DMSO, 400 MHz, δ 8.11–7.84 (m, 3H), 2.64 (s, 6H).

Surface plasmon resonance (SPR) analysis

Sensor gold slides were purchased from BioNavis. The chip surface was cleaned with H_2O:H_2O_2:NH_3-%25 (5 mL/1 mL/1 mL) at 80–90 °C for 10 minutes, the chip was rinsed multiple times with deionized water and the surface of the chip sensor was dried under a stream of nitrogen gas and used immediately after cleaning.

Binding kinetics were analyzed *via* Surface Plasmon Resonance (SPR) on a MP-SPR Navi™ 200 OTSO instrument. Binding was analyzed by measuring the changes in the refractive index

at an angular-scan angle at 670 nm. Binding of **CD-SH** to the surface was measured by capturing **CD-SH** (0.2 mM) by injection of 1 mL of this compound at a flow rate of 30 μL min^{-1} in H_2O : DMSO (95 : 5 v/v). Analytes were diluted in H_2O : DMSO (95 : 5 v/v) and 1 mL of the sample was injected at a flow rate of 30 μL min^{-1}. The association rate constant (k_a) represents the number of complex structures created each second. The dissociation rate constant (k_d) indicates the fraction of complexes that decay every second. SPR data were used to determine k_a and k_d. Finally, the equilibrium constant (K_D), which represents the ligand affinity for the molecule, was computed. The association/dissociation periods and concentration ranges were adjusted to fit the respective analytes. Binding kinetics (k_a, k_d and K_D) values were determined by global fitting of curves, assuming a 1 : 1 binding stoichiometry, using the Trace Drawer 1.10.1 software.

Dye displacement assay

Alizarin red S (ARS, 0.1 mM) and **CD-SH** (1 mM) were mixed in Phosphate Saline Buffer (PBS) containing 1% methanol. The mixture was stirred for 1 h at RT in the dark to enable the formation of **CD-SH** and ARS host–guest complexes. Then, aliquots of 0.2 M AHL molecules in DMSO (Scheme S1) were added to the initial solution and the fluorescence spectra was recorded by exciting the sample at 485 nm (Agilent Cary Eclipse). The change in the fluorescence value at 546 nm was monitored.

Cell culture

Pseudomonas aeruginosa PAO1 was cultured in nutrient broth (NB) media at 37 °C until an optical density at 600 nm (OD_{600}) of 1.0 was reached, which marked the starting point for all experiments. A549 (CCL-185™) cells were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium high-glucose (hDMEM), maintained at 37 °C in a humidified incubator with 5% CO_2. The cells were regularly monitored for confluency and passaged according to standard protocols to maintain healthy growth for subsequent experiments.

Minimum inhibitory concentration test (MIC)

To assess the effect of the CD derivatives on bacterial viability, a MIC assay was conducted with *P. aeruginosa*. A single pure colony was cultured overnight in NB at 37 °C, and a bacterial suspension of 1×10^6 CFU mL^{-1} was prepared. 5 μL of this suspension was added to each well of a 96-well plate, followed by the addition of various concentrations of the compounds. The plate was incubated at 37 °C for 24 h. After incubation, bacterial growth was measured by recording the optical density (OD) at 600 nm using a BioTek Epoch 2 microplate reader, allowing for the determination of the lowest concentration that inhibits growth.

Biofilm formation assay

For quantitative biofilm formation, *P. aeruginosa* (1×10^6 CFU mL^{-1}) was cultured by adding 5 μL to 100 μL of NB per well in a 96-well plate and incubated overnight at 37 °C. The experiment was conducted in the presence of 400 μM of CD, **CD–Cu(II)**, and various concentrations of **CD-SH**. After incubation, 125 μL of 0.1% crystal violet solution was added to each well and incubated at room temperature for 15 minutes. Afterward, the plate was washed four times and dried for 2 hours. To solubilize the crystal violet, 125 μL of 30% acetic acid was added to each well and incubated for 15 minutes. Then, 100 μL of the solubilized dye was transferred to a new flat-bottomed microplate. Biofilm formation was quantified using a BioTek Epoch 2 microplate reader® at 550 nm, with 30% acetic acid serving as the blank.

Pyocyanin extraction and quantification

Bacteria were incubated in NB with either 25 μM of **CD-SH** or 400 μM of CD and **CD–Cu(II)** for 24 hours. Pyocyanin was extracted from the culture supernatant by adding 500 μL of chloroform to 1 mL of supernatant, followed by vortexing for 1 minute. The samples were then centrifuged at 4 °C for 10 minutes at 10 000 rpm. The resulting blue layer at the bottom was carefully transferred to a new Eppendorf tube, and 250 μL of 0.2 M HCl was added, followed by vortexing. The samples were centrifuged again for 2 minutes at 10 000 rpm. Then, 100 μL of the pink layer was transferred to a 96-well microplate for absorbance reading using a BioTek Epoch 2 microplate reader® at 520 nm, with 0.2 M HCl serving as the blank. The pyocyanin concentration was calculated using the following formula:

$$[\text{Pyocyanin}]\ (\mu g\ mL^{-1}) = \text{sample absorbance}\ (A_{520}) \times 17.072$$

Gene expression analysis

RNA was extracted from *Pseudomonas aeruginosa* and A549 cells using the PureZol RNA isolation reagent (cat #7326890). Genomic DNA was removed by treating the samples with DNase-I (Thermo Scientific, EN0521), following the manufacturer's instructions. cDNA was synthesized using the iScript™ cDNA synthesis kit (Bio-Rad, 170-8891) according to the provided protocol. Gene expression changes were analyzed using a Real-Time PCR System (Bio-Rad CFX Connect™ Optics Module). Sequences of primers used in RT-PCR are given in Table S1. The PCR mix included SYBR® Green supermix (Bio-Rad, cat #1725270), and the thermal cycling program consisted of an initial denaturation at 95 °C for 30 seconds, followed by 39 cycles of primer annealing at 60 °C for 30 seconds, extension at 72 °C for 30 seconds, and a brief denaturation at 95 °C for 15 seconds. A final extension was performed at 95 °C for 30 seconds. The threshold cycle (CT) values for target genes and endogenous control genes (rpsL and/or proD for bacteria and β-actin for lung cells) were used to compare groups using the $2^{-\Delta\Delta Ct}$ method for relative gene expression analysis.

Mammalian lung cell toxicity analysis

The cytotoxic effect of **CD-SH** on A549 cells was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, a colorimetric method for assessing cell metabolic activity. A549 cells were seeded into 96-well plates at a density of 1×10^4 cells per well and allowed to adhere for 24 hours under standard culture conditions (37 °C, 5% CO_2).

Following this, the cells were treated with varying concentrations of **CD-SH** (starting from 3.125 to 400 μM mL^{-1}) for an additional 24-hour incubation period. Subsequently, the MTT reagent (0.5 mg mL^{-1}) was added to each well to assess cell viability. The MTT reagent was reduced by metabolically active cells to form insoluble formazan crystals, which were dissolved in dimethyl sulfoxide (DMSO, 100 μL). Absorbance was measured at 570 nm using a microplate reader, and cell viability was calculated as a percentage relative to untreated control cells using the following equation:

$$\text{Cell viability (\%)} = A_{570}\,(\text{treated cells})/A_{570}\,(\text{untreated cells}) \times 100$$

Co-culture analysis

A549 cells were seeded into 6-well plates at a density of 1×10^6 cells per well and allowed to adhere for 24 hours under standard culture conditions (37 °C, 5% CO_2). After this, the cells were treated with 5 μL of *P. aeruginosa* POI-1 (OD_{600} = 1.0) in HG-DMEM medium (without antibiotics) for 4 h. Following the treatment, 50 μg mL^{-1} of gentamicin and 25 μM of **CD-SH** were added, and the cells were incubated for an additional 24 h.

Statistical analysis

Data were expressed as mean standard deviation (SD), and each experiment was carried out in at least triplicate. Real-time PCR result data were analysed using GraphPad prism 9. Data were normalized to housekeeping genes. Student's *t*-test was used to compare between the control and treatment groups for all $2^{-\Delta\Delta Ct}$ data. On the other hand, 1-way or 2-way ANOVA was used to compare data. $P \leq 0.05$ was considered statistically significant.

Results and discussion

A β-CD dimer, CD, has been synthesised as a potential AHL binding scaffold using 6-monoamino-β-cyclodextrin and 2,6-bis(bromomethyl)pyridine.[12] The resulting compound is purified by dialysis. CD with Cu(II) for the catalytic core is prepared using $Cu(ClO_4)_2{\cdot}6H_2O$ and **CD–Cu(II)** is prepared to mimic an artificial holo-enzyme (enzyme bound to cofactor). The CD alone is considered as an apo-enzyme without any catalytic activity but can still have AHL binding.

To determine the lactonase activity of the artificial system, QS modulator *N*-hexanoyl-DL-homoserine lactone is used as a model substrate. ^{1}H Nuclear Magnetic Resonance (NMR) analysis with and without the 4% **CD–Cu(II)** load (4 μg mL^{-1}) is analysed. Following 20 h of incubation, AHL is shown to be hydrolysed with 35% calculated efficiency (Fig. 3). Lactone core protons resonating at 4.50 (H_a), 4.30 (H_{b1}) and 4.15 (H_{b2}) ppm shift to 4.8 and 3.6 ppm upon hydrolysis consistent with the literature.[13] AHL conversion per **CD–Cu(II)** is determined to be more than 8 (8.25 AHL/**CD–Cu(II)**), indicating a catalytic chemical conversion. Hydrolytic products were not detected with **CD-SH** which might be due to the poor thioester hydrolysis rate under in-tube experimental conditions.

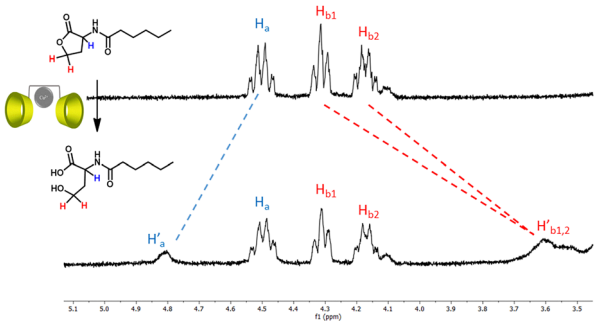

Fig. 3 Hydrolysis of *N*-hexanoyl-DL-homoserine lactone by **CD–Cu(II)**. ^{1}H NMR spectra of AHL alone (top) and AHL in the presence of 4% **CD–Cu(II)** (4 μg mL^{-1}) after incubation for 20 h (400 MHz, d-DMSO).

The effect of CDs on the modulation of AHL-regulated pathways in *Pseudomonas aeruginosa* PAO1 strain is investigated. This bacterium is responsible for most of the hospital-acquired infectious diseases and respiratory infections in cystic fibrosis (CF) patients and uses AHL-dependent chemical communication.[14] Pyocyanin is an exotoxin produced by the bacteria, causing CF pathogenesis, and its production is regulated by QS.[14] Pyocyanin is shown to induce inflammatory response through interfering with cytokine and mucin hypersecretion in the CF host which induces bacterial accumulation, neutrophil recruitment and pulmonary dysfunction.[14c] *P. aeruginosa* was treated with 400 μM **CD–Cu(II)** or the metal-free CD for 24 h. Metal free form of the molecule is not expected to catalyse AHL hydrolysis but can encapsulate AHL molecules, so serves as a negative control group. Pyocyanin was extracted from the bacterial cell culture and absorbance at 520 nm was monitored.[15] As shown in Fig. 4, the CD and other control compounds have no effect on pyocyanin production, whereas metal bearing **CD–Cu(II)** displayed a significant decrease in the level of exotoxin. Likewise, 25 μM **CD-SH** treatment leads to a 27% decrease in the pyocyanin level. These results suggest the contribution of enzyme mimetic units (metal and thiol) to pyocyanin inhibition.

Knowing that any agent having a lethal or growth inhibition effect generates a selective pressure and favours the

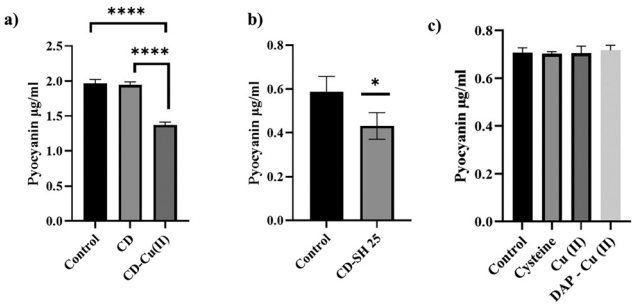

Fig. 4 Effect of CD derivatives on *P. aeruginosa* pyocyanin production when treated with (a) **CD–Cu(II)** (400 μM), CD (400 μM); (b) **CD-SH** (25 μM); (c) **Cu(II)** (Cu $(ClO_4)_2{\cdot}6H_2O$, 400 μM), DAP-Cu(II) (400 μM) and cysteine (25 μM) (n = 3; *$p \leq 0.05$; ****$p \leq 0.0001$). Quorum sensing dependent pyocyanin production cannot be inhibited by CD which lacks enzyme mimetic catalytic units (Cu^{2+} or thiol).

development of resistant species, the effect of CDs on the viability of the *P. aeruginosa* was tested.[16] *P. aeruginosa* viability did not change significantly at tested doses (Fig. S2). Therefore, rather than killing the bacteria, compounds inhibit the pathogenic processes at the application doses. **CD-SH** also reduced biofilm formation up to 41% at a concentration as low as 12.5 μM (Fig. S3). The biofilm inhibitory effect of the compound decreases at 400 μM. At this concentration, the compound also displays a bactericidal effect. Therefore, it is likely that the compound starts to interfere with additional pathways or cellular targets, leading to a change in response.

AHL is known to modulate the pathogenic phenotype of bacteria through changing the expression of QS genes including the ones responsible for quorum signal production, perception and signal transduction. For *P. aeruginosa*, *LasR*, *LasI*, *rHII* and *rHIR* are among those genes that are involved in pyocyanin production and many other processes such as virulence and biofilm formation.[17] To understand the effect of CDs, the bacterium is incubated with the compounds for 24 h at 37 °C. Isolated RNA is converted into cDNA and quantified by RT-PCR. The results indicate a significant increase in *rhIR* gene expression both in **CD–Cu(II)** and **CD-SH** but not CD treated samples (Fig. 5). Considering that *rhIR* is an AHL receptor, the **CD-SH** and **CD–Cu(II)** mediated increase in the expression is attributed to adaptive behaviour of the bacteria to respond to lower AHL levels. Interestingly, **CD–Cu(II)** elevated the *rhII* expression whereas **CD-SH** reduced the level of this gene responsible for butyryl homoserine lactone production. Similarly, the increase in the expression of *rhII* upon **CD–Cu(II)** treatment might be a response to lower AHL levels. The major effect of **CD–Cu(II)** seems to induce an adaptive response to the elevated production and reception of AHLs, whereas **CD-SH** additionally displays significant quorum quenching through reduction of *LasR*, *LasI* and *rHII* expression at much lower concentrations (25 μM).

A model compound DAP–Cu(II) was prepared by forming a complex between 2,6-diacetyl pyridine and Cu(II) and it was applied to bacteria. As a thiol model, cysteine is used since various other model thiols turned out to be cytotoxic to lung cells (*i.e.* beta-mercaptoethanol). The results indicate that these models do not induce the same response (Fig. 5c). When quorum sensing genes are considered, models induce a decrease only in RhIR gene expression; however, the decrease is not statistically significant. Other than that, bacterial QS gene expression has not changed.

Major reasons for pulmonary dysfunction in CF patients are over-stimulation of inflammatory responses and inability to clear the pathogens from the lungs. Overproduction of pro-inflammatory cytokines recruits neutrophils and hypersecretion of mucin persists bacterial attachment and infection.[18] For this reason, immunological modulation such as the use of drugs having an anti-inflammatory effect is one of the promising therapeutic approaches. To understand host response of **CD-SH**, A549 human lung cancer cells are co-cultured with *P. aeruginosa* for 4h and the viability and inflammatory response of lung cells are analysed. Infecting the lung cells

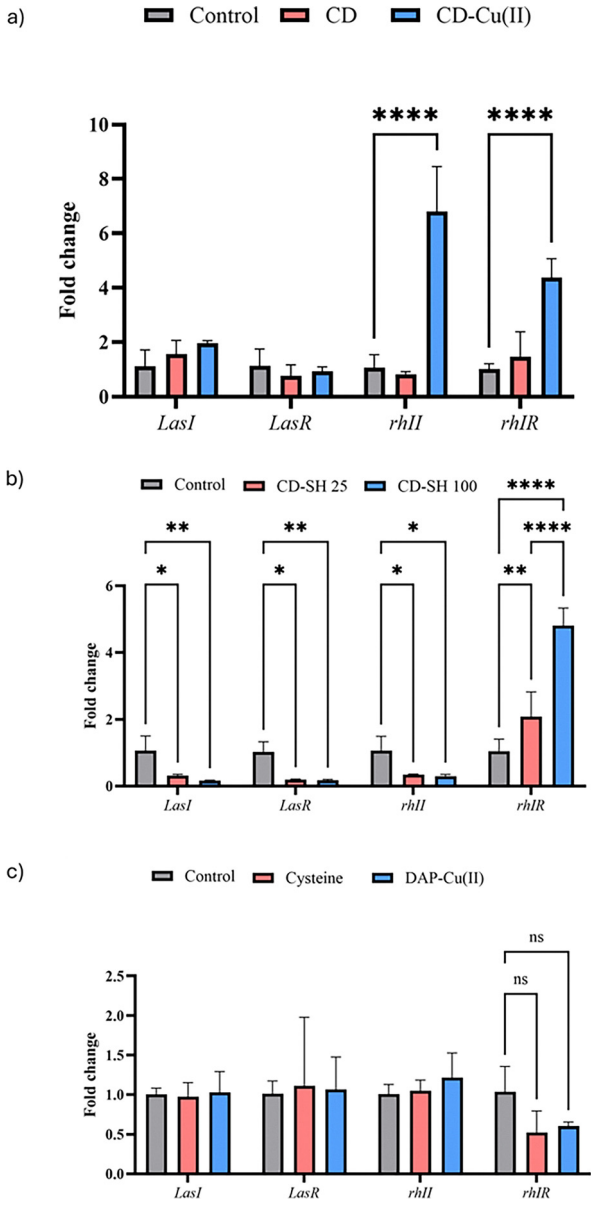

Fig. 5 Relative mRNA levels of *P. aeruginosa* QS genes upon treatment with (a) **CD–Cu(II)** (400 μM), CD (400 μM); (b) **CD-SH** (25 μM); (c) **DAP–Cu(II)** (400 μM), and cysteine (25 μM) ($n = 3$; *$p \leq 0.05$; **$p \leq 0.01$; ****$p \leq 0.0001$); *rpsL* and *proD* are used as internal housekeeping reference genes. The control group corresponds to untreated *P. aeruginosa*.

with the bacteria caused a 71% decrease in lung cell viability (Fig. 6e). **CD-SH** is non-toxic to lung cells and the viability of infected lung cells is restored significantly when the culture media are supplemented with 25 μM of **CD-SH**, demonstrating the cytoprotective effect towards host cells during infection (Fig. 6e and Fig. S4). PCR analysis indicates that proinflammatory cytokine and mucin gene expressions is increased dramatically in the presence of bacterium (Fig. 6a–d). **CD-SH** treatment significantly reduced IL-1, IL-6, IL-8 and MUC5B levels compared to bacterial infection alone. These cytokines play critical roles in the inflammatory response by recruiting immune system elements to combat pathogens. Excessive activation of IL-1β, IL-6, and IL-8 during

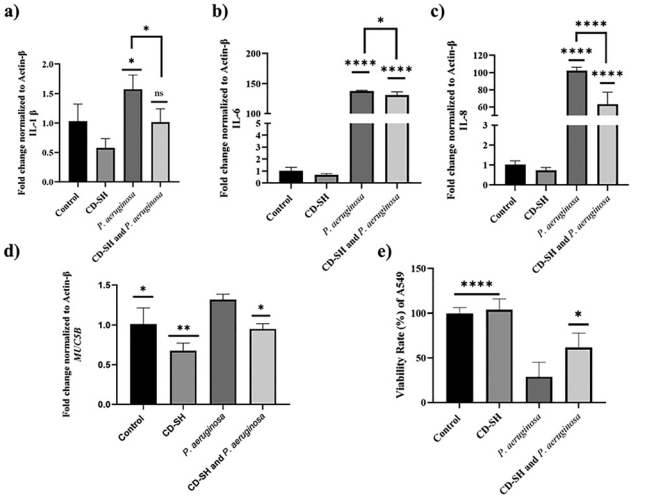

Fig. 6 Effect of *P. aeruginosa* and **CD-SH** (25 μM) on mRNA levels (a–d) and viability (e) of A549 human lung cells (β-actin is used as reference gene, $n = 3$; ***$p < 0.001$; ****$p = <0.0001$). **CD-SH** displays a cytoprotective role in infected lung cells together with reduction in immune response. The control group refers to untreated and uninfected A549 cells. All other experimental groups contain A549 cells treated with **CD-SH** and/or infected with the bacteria.

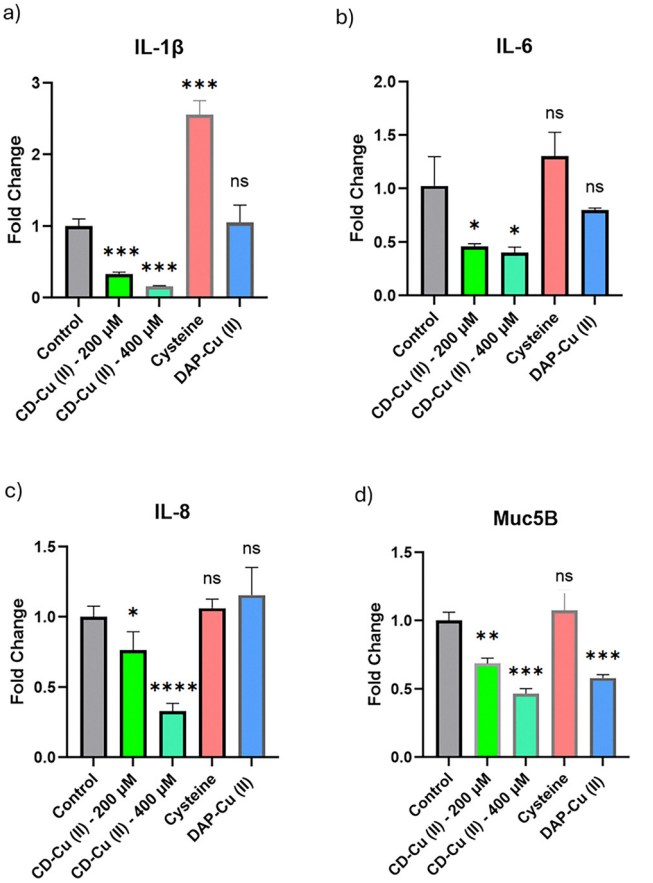

Fig. 7 Relative mRNA levels of (a) IL-1β; (b) IL-6; (c) IL-8; (d) Muc5B in *P. aeruginosa* infected A549 cells when treated with **CD–Cu(II)** (200 μM or 400 μM), cysteine (25 μM) or DAP–Cu(II) (400 μM) ($n \geq 3$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$); β-actin is used as reference gene. The control group refers to untreated infected A549 cells. Other groups are infected A549 cells treated with the given compounds.

infections can lead to a cytokine storm, resulting in widespread inflammation and tissue damage, which can potentially cause organ failure and severe sepsis.[18a] Reduction of cytokines and mucin by **CD-SH** during infection indicates its potential for anti-inflammatory and mucin reduction treatment of CF.[18b] Expression of interleukins in uninfected lung cells is unchanged in the presence of **CD-SH**. Therefore, the cytoprotective role of **CD-SH** is attributed to the reduced immune response because of quorum quenching.

CD–Cu(II) displays an even better decrease in IL-1β, IL-6, IL-8 and Muc5B expression of infected lung cancer cells (Fig. 7). At 400 μM, 84%, 59%, 67% and 54% decreases in expression are observed, respectively. To demonstrate that the CD binding pocket connected to Cu(II) or thiol units is essential for the observed biological response, control experiments were performed.

The DAP–Cu(II) model compound does not affect interleukin 1β, 6 and 8 expressions in infected lung cancer cells (Fig. 7). Muc5B expression is decreased by DAP–Cu(II), but the decrease is not as effective as that done by **CD–Cu(II)**. Therefore, Cu(II) might contribute to the decrease in MUC5B gene expression. Similarly, the cysteine thiol model does not show any change in IL6, 8 and Muc5B, but an increase is observed in IL-1β. **CD-SH** leads to a decrease in IL-1β. Together with the results obtained by unfunctionalized CD, findings suggest that CD or thiol/Cu(II) alone does not display the same immunogenic response.

Dye displacement assay and SPR analysis were performed to analyse the binding of the AHLs to the cyclodextrin cavity. For this purpose, three different AHL molecules having different hydrophobic tails (*N*-butyryl-L-homoserine lactone, *N*-hexanoyl-L-homoserine lactone and *N*-octanoyl-L-homoserine lactone) were synthesised (Scheme S1). **CD-SH** was saturated with alizarin red S (ARS) dye to form a host–guest complex as described in the literature.[19] Addition of AHL aliquots leads to the displacement of ARS and fluorescence intensity is reduced (Fig. S5). Comparison of the relative change in emission indicated a better binding of long alkyl chain AHL. SPR analysis of **CD-SH** titrated with *N*-hexanoyl-L-homoserine lactone estimates the dissociation constant (K_D) to be 3.99×10^{-5} M (Fig. S6).

Conclusions

Scientists working in the field of catalysis and biomimetic nanomaterials are usually inspired by biology and its evolutionary strategies.[20] Here, CD derivatives as artificial enzyme models mimicking metalloproteins and cysteine lactonases are shown to modulate bacterial communication and reduce bacterial toxicity to host human lung cells. The hydrophobic cavity of the β-CD provides a binding pocket for AHL. This encapsulation was previously explored by others using CDs without metals or thiols.[7] For the approximately same fold reduction of the *P. aeruginosa* pyocyanin level, 5 mM β-cyclodextrin was reported to be necessary, which is approximately 12 and 192

times more than the concentration used in this study for **CD–Cu(II)** and **CD-SH**, respectively.[7d] Therefore, supramolecular encapsulation alone has very poor quorum quenching ability. Here, we have shown that the presence of divalent metals or thiols in the vicinity of AHL enables reduction in pyocyanin and modulates QS associated gene expression at a much lower concentration proposed to be due to catalytic degradation rather than just supramolecular encapsulation. NMR analysis indicates that each **CD–Cu(II)** can convert more than 8 AHL molecules to inactive carboxylate form, which explains the difference between metal bearing **CD–Cu(II)** and naked CD in pyocyanin inhibition efficiency. Solution analyses with **CD-SH** do not show clear evidence of AHL hydrolysis; however, this compound displays the same reduction in pyocyanin production and even much better quorum quenching. Considering the inefficiency of CD, encapsulation cannot be the only reason for quorum quenching for **CD-SH**. Under cellular conditions, hydrolysis may take place or additional thiol mediated changes might be involved. The exact mechanism of QS and immune response modulation should be carefully studied.

When lung cells are infected with *P. aeruginosa*, a dramatic increase in cytokine is observed. There are clinical and scientific evidence/observations that lung cancer tissue mimics pulmonary fibrosis. Indeed, tumour suppressor protein PTEN is involved in the anti-*Pseudomonas aeruginosa* immunity.[21] Therefore, in this work, lung cancer cells are used to understand the response of the CD derivatives. Cytokines are known to be involved in CF pathogenesis by its effect on immune stimulation and undesired pulmonary inflammatory response. Mucins, large glycoproteins, are responsible for bacterial attachment and accumulation. **CD-SH** and **CD–Cu(II)** are shown to reduce the level of IL-1β, IL-6, IL-8 and *MUC5B* and hence likely to show an anti-inflammatory therapeutic effect in CF. Considering that **CD-SH** has no effect on the expression of interleukins in uninfected lung cells, observed immune modulation is likely to result from quorum quenching. Reduced biofilm formation enables the reach of the antibacterial agents to the target pathogen; hence **CD-SH** has promising potential to be used as an antipathogenic agent.

Free thiols can interfere with redox chemistry of the cell. Therefore, **CD-SH** may contribute to reducing reactions of the cell, *i.e.* disulfide cleavage. However, the response of **CD-SH** is different from that of CD and cysteine, indicating that neither a free thiol nor the CD cavity is solely responsible for the observed immunomodulatory results. The results might be a synergistic effect of both. Further research can shed light on the exact mechanism of cellular response.

The requirement of high **CD–Cu(II)** concentration for effective cellular response may be due to coordination of cellular metabolites to copper (II). Shielding of the Cu(II) centre might avoid untargeted binding; however, this might also interfere with the binding of AHLs. Careful molecular engineering might be done to improve the activity of the compound.

This study demonstrates the quorum modulation of bacteria and immune modulation of the host by supramolecular cyclodextrin structures. The effect of the CD derivatives on different immunomodulatory QS molecules, *i.e.* *N*-3-oxo-dodecanoyl-L-homoserine lactone, can be studied to understand the exact cellular mechanism and/or targets. Modifications of the cavity would allow selection among AHLs and regulate communication of certain bacteria only. This study may pave the way for the expansion of biological use of biomimetic supramolecular enzyme models in quorum and immune modulation.

Author contributions

S. A. and N. G. O. contributed equally. S. A. and N. G. O. performed microbiology experiments, N. G. O. synthesized the compounds and did NMR experiments. S. E. C. proposed and coordinated the research. SPR binding constant analysis was performed by E. K. The authors acknowledge Prof. Dr Nurettin Menges for allowing the use of the SPR device of his laboratory.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data used in this article are available at **https://drive.google.com/drive/folders/1tIzu7ppIfxeJXAL5-_LFmlTVo1XUYxDm?usp=drive_link**.

Additional experimental details and figures are available in supplementary information. See DOI: **https://doi.org/10.1039/d5ma00162e**

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