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Alkali–earth metal bridges formed in biofilm matrices regulate the uptake of fluoroquinolone antibiotics and protect against bacterial apoptosis

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Abstract

Bacterially extracellular biofilms play a critical role in relieving toxicity of fluoroquinolone antibiotic (FQA) pollutants, yet it is unclear whether antibiotic attack may be defused by a bacterial one-two punch strategy associated with metal-reinforced detoxification efficiency. Our findings help to assign functions to specific structural features of biofilms, as they strongly imply a molecularly regulated mechanism by which freely accessed alkali–earth metals in natural waters affect the cellular uptake of FQAs at the water-biofilm interface. Specifically, formation of alkali-earth-metal (Ca$^{2+}$ or Mg$^{2+}$) bridge between modeling ciprofloxacin and biofilms of Escherichia coli regulates the trans-biofilm transport rate of FQAs towards cells (135-nm-thick biofilm). As the addition of Ca$^{2+}$ and Mg$^{2+}$ (0–3.5 mmol/L, CIP: 1.25 µmol/L), the transport rates were reduced to 52.4% and 63.0%, respectively. Computational chemistry analysis further demonstrated a deprotonated carboxyl in the tryptophan residues of biofilms acting as a major bridge site, of which one side is a metal and the other is a metal girder jointly connected to the carboxyl and carbonyl of a FQA. The bacterial growth rate depends on the bridging energy at anchoring site, which underlines the environmental importance of metal bridge formed in biofilm matrices in bacterially antibiotic resistance.

1. Introduction

More and more attention has been paid to the challenging issue on environmental contamination of antibiotics in recent years (Martinez, 2009; Pruden et al., 2006). A nationwide survey of pharmaceutical compounds from USA showed that a number of antibiotics were detected in 27% of 139 rivers at concentrations up to 0.7 µg/L (Kolpin et al., 2002). And the annual usage of antibiotics has been estimated to be between 1.0 × 10^5 and 2.0 × 10^5 tons globally, with more than 2.5 × 10^4 tons used each year in China (Xu et al., 2007). The cellular uptake of antibiotics via trans-biofilm transport will inevitably increase the risk of microbial death, ecological disruption, amplification of antibiotic resistance genes and even the creation of “superbugs” (Desnottes and Diallo, 1992; Pruden et al., 2013). However, regulated mechanism occurring at water-biofilm interface, by which bacteria exert extracellular biofilm barrier to defuse the antibiotic stress, has not been well understood. This process has profound consequences for environmental stability.

Fluoroquinolone antibiotics (FQAs), which comprise an important and hard-degradable class of synthetic pharmaceuticals, have been widely used and have been introduced into the environment by a multitude of human and veterinary activities over the last 30 years in Europe and the United States (Baquero et al., 2008; Mompelat et al., 2009). Bacteria generally hide into a biofilm to deal with the presence of these synthetic antibiotics (Wingender et al., 2012). One component of the so-called bacterial one-two punch strategy is to use an extracellular biofilm permeability barrier to impede the cellular uptake of antibiotic stressors. Such physical defenses in response to antibiotics might be...
inefficient and energetically very costly (Martínez et al., 2007; Rice, 2006). For example, previous studies have shown that sorption of FQAs to oxygen- and nitrogen-containing dissolved organic matter (DOM) displays a nonlinear saturation pattern (with a Freundlich exponent $n = 0.40–0.51$) (Zhang et al., 2012), whereas their affinity to phospholipids exhibits a linear partitioning pattern (partitioning coefficient $K_{ow} = 20.0–79.4$ L/kg) (Merino et al., 2002, 2003). These findings imply that biofilms containing similar oxygen and nitrogen groups have a relatively low capacity (Walter, 2012) to absorb FQAs and may result in high intracellular uptake due to the low-efficiency of extracellular FQA interception. Furthermore, extracellular accretions that act as entrapment agents will be energetically very costly, considering the demand for major saccharide and protein moieties during the formation of biofilms. Nevertheless, little information is currently available to determine how bacteria efficiently and economically deal with such stressors; this issue requires proper investigation.

A positively charged metal bridging connection between electronegative biofilms and negatively charged FQAs (i.e., their functional groups) may be the other component of the one-two punch strategy for defending against antibiotic attack. Given the strong electrostatic attraction between electronegative biofilms/FQAs and accessible alkali–earth metals, the formation of a stable structure would anchor the FQAs in biofilms via a metal bridge. Whereas one side of the metal-to-FQA connection (metal–carbonyl interaction) is supposedly understood (Aristilde and Sposito, 2008), attractions between metals and extracellular biofilm components on the other side are more multifarious and complicated. Thus, a quantitative description of an alkali–earth–metal bridge based on these weak attractions is crucial for quantitatively expounding the association of such weak interactions at the molecular level. Generally, on the basis of spectroscopic data, it is considered that alcohol, carboxyl, phosphoric, and amino-acid residue groups in biofilm matrices may be involved in interactions with such metals as $\text{Zn}^{2+}$, $\text{Co}^{2+}$, and $\text{Ca}^{2+}$ (Ha et al., 2010; Sun et al., 2009; Sundararajan et al., 2011; Xiong et al., 2002). These results only suggest that biofilms may utilize their electronegative groups to interact with metals, but not know whether antibiotic attack may be defused by a metal-reinforced detoxification efficiency occurring at water-biofilm interface. And these spectroscopic data should be considered qualitative, as they do not specify which of the functional groups in biofilms are involved in metal binding. The listed spectroscopic techniques have been unable to access quantitative descriptions of imperceptibly weak interactions at the microscopic level. Therefore, based on the idea that FQA–biofilm interaction occurs via a metal bridge, including bridging energy and spectroscopic analyses, multiple approaches are necessary to substantiate an association between alkali–earth metals and the extracellular retardation of FQAs and biological growth.

In the present study, we investigated the association of bacterial extracellular biofilms with FQAs via an alkali–earth–$\text{Ca}^{2+}/\text{Mg}^{2+}$ bridge and determined the influence of metal bridges in biofilms on the extracellular interception and cellular uptake of FQAs. In situ confocal laser scanning microscopy (CLSM), X-ray photoelectron spectroscopy (XPS), Fourier transform infrared spectroscopy (FTIR), and $^{13}$C nuclear magnetic resonance ($^{13}$C NMR) were used to confirm the formation of alkali–earth–metal bridges in biofilms. A series of batch experiments was performed to explore the effect of alkali–earth–metal bridges in biofilm matrices on extracellular interception, trans-biofilm cellular uptake of a model antibiotic, and bacterial apoptosis. Subsequently, eight FQAs were used to determine the dependence of $E.\ coli$ growth on the bridging energy at major sites through a set of fluorescence microtitration and computational chemistry analyses.

2. Materials and methods

2.1. Materials

Eight fluoroquinolone antibiotics (FQAs, $\geq 99.0\%$) were purchased from Sigma-Aldrich Co., Ltd, USA, namely ciprofloxacin (CIP), norfloxacin (NOR), enrofloxacin (ENR), ofloxacin (OFL), lomefloxacin (LOM), levofloxacin (LEV), pefloxacin (PEF), and fleroxacin (FLE). Their physicochemical properties, including molecular weight (MW), the dissociation constant for carboxyl groups ($pK_a,\text{carboxyl}$), and the octanol–water partition coefficient ($\log K_{ow}$), are listed in Table S1. Magnesium sulfate ($\text{MgSO}_4 \cdot \text{H}_2\text{O} \geq 99.0\%$) and calcium sulfate ($\text{CaSO}_4 \geq 99.0\%$) were purchased from Sinopharm Chemical Reagent Co., Ltd, China. Milli-Q water (18.2 MΩ × cm, Millipore, USA) was used for all experiments. Other chemicals (as listed below, purity > 99.0%) that were purchased from Nanjing Chemical Reagent Co., Ltd. were used to prepare modified chloride-free culture medium (Kang et al., 2014).

The medium contained $\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (28 mmol/L), $\text{KCl}$ (30 mmol/L), $\text{NaH}_2\text{PO}_4$ (2.2 mmol/L), $\text{NH}_4\text{NO}_3$ (18.7 mmol/L), $\text{CaSO}_4$ (0.001 mmol/L), $\text{K}_2\text{SO}_4$ (2.0 mmol/L), $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ (1.0 mmol/L), peptone (10 g/L), and a trace element solution (10 mL/L). The trace element solution contained $\text{Na}_2\text{EDTA}$ $\cdot \text{H}_2\text{O}$ (5.0 g/L), $\text{Fe}_{2}(\text{SO}_4)_{3}$ (0.37 g/L), $\text{ZnO}$ (0.05 g/L), $\text{CuSO}_4 \cdot \text{H}_2\text{O}$ (0.015 g/L), $\text{Co(NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (0.01 g/L), $\text{MgSO}_4$ (0.5 g/L), $\text{NiCl}_2$·6$\text{H}_2\text{O}$ (0.01 g/L), and $\text{H}_3\text{BO}_3$ (0.01 g/L). The pH of the medium was adjusted to 7.4 with sulfuric acid (98.0%). When necessary, solid agar medium was prepared with 1 L of chloride-free medium and 15 g of agar powder. The sulfuric acid (0.01 mmol/L/$\text{H}_2\text{SO}_4$) and sodium hydroxide (0.02 mmol/L/$\text{NaOH}$), used for pH adjustment in batch experiment and fluorescence microtitration, were purchased from Nanjing Chemical Reagent Co., Ltd. (Nanshi, China).

2.2. Biofilm manipulation

Our previous method was used to manipulate the extracellular biofilms (Fang et al., 2002; Kang et al., 2014; Liu and Fang, 2002). In brief, Escherichia coli (DH5α) was initially cultured in 20 mL of chloride-free medium at 37 °C for a 12-h recovery of growth. The bacterial suspension (5 mL) was then transferred to fresh chloride-free medium ($1.0 \times 10^3$ mL) and grown for an additional 48 h to reach the stable growth phase. E. coli cells were collected by low-speed centrifugation ($3 \times 10^2$ g, 6 min, 4 °C) followed by washing with Milli-Q water to obtain pure, pristine E. coli (high-biofilm E. coli).

Low-biofilm E. coli was obtained by the removal of biofilms from the E. coli surface using a sonication/centrifugation procedure (Kang et al., 2014). Specifically, one-half of the (high-biofilm) E. coli pellet was suspended to an initial volume of 500 mL and then processed by a low-intensity ultrasonic process for 7.0 min at an intensity of 2.5 W/cm² and a frequency of 40 KHz at 4 °C. The E. coli suspension was then centrifuged for 20 min at $2.0 \times 10^4$ g and 4 °C. The settled pellets were collected as low-biofilm E. coli samples. The supernatant, which was filtered through a 0.22-μm membrane (Anpel, Shanghai, China), represented an aqueous biofilm solution and was stored at 4 °C for later chemical analyses. Elemental analysis was performed on freeze-dried biofilms using an X-ray photoelectron spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), and the percentage contents of C (38.96), O (29.88), N (5.67), and $\text{S}$ (1.02) were determined. The dry weight of the extracted aqueous biofilms ($25.7$ mg/L) was measured by an oven-drying method (2 h at 105 °C) (Comte et al., 2006). The major proteins (345.1 mg/g) and polysaccharides (173.8 mg/g), and a small quantity of nucleic acid (0.33 mg/g), were measured according to previously reported techniques (Burton, 1956; Dubois et al., 1955; Falbe et al., 1980).
et al., 1956; Lowry et al., 1951). The low concentration of nucleic acid in the biofilms indicated negligible cell lysis during the biofilm extraction procedure.

2.3. Interception and uptake of FQA and E. coli apoptosis

High- and low-biofilm E. coli cells that were subjected to different biofilm manipulations were suspended in 1.0 L of ultrapure water to obtain approximately $4.0 \times 10^5$ cells/mL of each type of E. coli. A liquid suspension of bacteria (40 mL) was placed into a 50-mL conical flask equipped with a permeable silica-gel stopper prior to the addition of aqueous alkali–earth–metal stock solutions (200 mmol/L, alkali–earth Ca$^{2+}$ or Mg$^{2+}$) and an aqueous CIP stock solution (1 mmol/L) to the desired concentrations (metals: 0–3.5 mmol/L; CIP: 1.25 mmol/L). Notably, the concentrations of the added alkali–earth metals were within the range of natural oceanic water (Ca$^{2+}$: 53.75 mmol/L; Mg$^{2+}$: 10.1 mmol/L) and fresh water (Ca$^{2+}$: about 1.75 mmol/L; Mg$^{2+}$: about 1.77 mmol/L) (Adkins et al., 2002; Bowen, 1979). Here, the added concentration of CaSO$_4$ was far below its solubility limit (14.2–15.4 mmol/L at 10–40 °C) (Harvie et al., 1984). The static suspension experiment was performed in the dark for 4 h at 120 rpm and 37 °C.

A portion of the E. coli suspension was used to extract the CIP that was retained in extracellular matrices and absorbed into the cell interiors. The CIP in extracellular matrices was extracted using the biofilm-extraction method described above. The CIP antibiotic was obtained during the separation of biofilms from the E. coli surface. For low-biofilm E. coli, CIP extraction from extracellular matrices was also performed using the same procedures. Additionally, an ultrasonic method of cellular disruption was used to obtain the intracellular components for CIP antibiotic quantification (Turiel et al., 2006). An E. coli sample (5 mL) was subjected to ultrasonic disruption at 450 W and 24 kHz (Q6, Scientz, China). The period was 2.0 s with a 2.0-s interval in an ice-water bath for 5 min, unless otherwise indicated. No bacterial colonies were observed in a plate test (15 g/L agar in chloride-free medium), which suggested that the E. coli cells were completely disrupted by the high-intensity ultrasound.

The CIP antibiotic in both extracted biofilms and disrupted E. coli cells was treated with a vacuum freeze-drying technique (−65 °C) that was followed by extraction using acetonitrile (7.0 mL) with a 1% formic acid-water solution (0.5 mL) (Johnston et al., 2002). The solutions were processed in a vortex mixer for 5 min and then centrifuged at 6000 g and 4 °C. This process was performed thrice, and the supernatants were pooled for later chromatographic analysis. The CIP in the supernatant was analyzed by high-performance liquid chromatography (HPLC) in an instrumental setup that included a 20-mL automatic liquid sampler, a Zorbax XDB-C18 column (Agilent, USA), and a fluorescence detector (FLD) (1260 series: Agilent, USA). The composition of the mobile phase was constant 87/13, v/v, acetonitrile/aqueous H$_3$PO$_4$ buffer (0.025 mmol/L; pH 3.0, adjusted with triethylamine). The FLD was operated at 278-nm excitation and 440-nm emission. The experiment demonstrated that the sequential method, including the CIP extraction and HPLC analysis, gave a high recovery of approximately $98 \pm 3.5\%$, indicating no loss or metabolism of CIP in the static suspension experiments.

After the static suspension experiment, another independent experiment was performed to investigate E. coli growth. A portion of E. coli from the static suspension experiment was inoculated into fresh chloride-free medium (40 mL; 4.0 × 10$^7$ cells/mL) and incubated for 18 h at 37 °C and 160 rpm. The cell densities (reflecting E. coli growth) were determined by a calibration curve for optical density [cell density (cell/mL) = (1.89 × OD$_{600nm}$ - 0.01) × 10$^8$], as described in a previous study (Kang et al., 2014).

2.4. Spectroscopic analyses of alkali–earth–metal bridges

CLSM technology was used to observe in situ the enhanced FQA–biofilm association via metals. A water-immersion lens (1000×) was used to observe the sample (50 μL) on a slide. Image stacks were created with a Zeiss LSM510 META that was controlled with AIM software (Jena, Germany). The fluorescence signal of the CIP was recorded with an excitation/emission matrix of 278/440 nm. The fluorescence signal of biofilms at this excitation/emission matrix could not be detected, but the signal from CIP attached to biofilms was clearly visible. XPS analysis was performed to probe the Ca$^{2+}$p and Mg$^{2+}$ signals from the bridges that were formed in E. coli biofilms (Thermo, USA). The sampling depth was less than 10 nm, suggesting that the sampling site was located in the biofilms and not on the cells (the biofilm thickness was approximately 135 nm).

TEM was used to observe the removal of the biofilm matrix from the E. coli surface. Dried E. coli cells were placed onto carbon-coated copper grids for TEM observation with a brightfield detector on a JEM-2100 (Tokyo, Japan). Additionally, after the static suspension experiment, the CIP-containing biofilms that had been extracted from the E. coli surface were also dried at −65 °C. FTIR (Buker, USA) and 13C NMR (Bruker, Germany) analyses were performed to characterize the functional groups of these dried biofilms through using previous method (Kang et al., 2014; Kang and Zhu, 2015).

2.5. Fluorescence micro titration

Three-dimensional excitation-emission matrix (3DEEM) fluorescence spectroscopy combined with micro titration (Kang et al., 2010, 2015) was used to quantitatively explore the association of FQA with the amino acid residues of biofilms in the presence of alkali–earth metals. In this test, the fluorescence peaks of tryptophan (Trp) residues in biofilms, which were located at 280/340 nm (EX/EM) in the 3DEEM, were traced by continuous microtitration/detection processes (Chen et al., 2003; Kang et al., 2010). Specifically, a CaSO$_4$ or MgSO$_4$ stock solution (10 mmol/L) was added in advance of the 20-μL aqueous biofilm solution (0.5 mg/L, dry weight basis) to obtain a 250 μmol/L Ca$^{2+}$ or Mg$^{2+}$ solution. Afterwards, an aqueous CIP stock solution (10 mmol/L) was titrated into each aqueous biofilm solution containing Ca$^{2+}$ or Mg$^{2+}$ using a chromatographic injector (25-μL scale, Agilent, Santa Clara, CA, USA); this step was followed by magnetic stirring for 20 min at 160 rpm, pH 7.0, and 25 °C. The fluorescence spectrum was recorded at an excitation wavelength of 200–370 nm (5-nm bandwidth) and an emission wavelength of 280–500 nm (5-nm bandwidth) at a speed of 3000 nm/min (F96PRO, Lengguang). These microtitration/detection procedures were repeated until there was no significant change in the fluorescence intensity. To obtain comparative data, similar procedures were also performed to explore the association between CIP and the biofilm matrix in the absence of alkali–earth metals. The fluorescence peak from extracellular biofilms matrix (EX/EM: 280/340 nm) did not overlap with the FQA peak (EX/EM: 278/440 nm). Thus, the changes in the fluorescence intensity of Trp residues in the biofilms that were caused by the CIP antibiotic could be well described by the relationship between the fluorescence intensity and the quencher concentration (Boaz and Rollesson, 1950; Eftink, 1997; Lakowicz and Weber, 1973). The Stern–Volmer equation is given as follows (Valeur and Berberan-Santos, 2012):

$$F_0 / F = 1 + K_Q [Q] = 1 + K_{SV}[Q]$$

where $F_0$ and $F$ are the relative fluorescence intensity of the
chromophore in the absence and presence of the quencher, respectively; \( K_q \) is the bimolecular quenching rate constant; \( \tau_0 \) is the average lifetime of the fluorophore in the absence of the quencher; \( [Q] \) is the concentration of the quencher; and \( K_{SV} \) is the Stern–Volmer quenching constant. For the static quenching process, equation (2) was used to determine the association constant \( (K_A) \) and the number of binding sites \( (n) \) (Bi et al., 2004). The association constants with biofilms via metal bridging were determined for all eight FQAs as follows:

\[
\log[(F_0 - F)/F] = \log K_A + n \log [Q].
\]

(2)

2.6. Quantum mechanical/molecular mechanical (QMM/MM) modeling

Utilizing Gabedit software (Version 2.4.5) (Allouche, 2011), we structured the 20 standard amino acid residues: alanine, leucine, isoleucine, valine, proline, phenylalanine, methionine, tryptophan, glycine, serine, glutamine, threonine, cysteine, asparagine, tyrosine, aspartic acid, glutamic acid, lysine, arginine, and histidine. These residues were docked with the CIP antibiotic via an alkali–earth–metal bridge. The key bridging site in biofilms must meet two necessary conditions: a high abundance in biofilms and a high bridging energy between the residue-metal and the FQA. First, the backbones of oligopeptides were composed of the reported seven most common amino acids in bacterial biofilms (Phe, Gly, Asn, Gin, Ser, Thr, and Tyr) (Nakashima and Nishikawa, 1992, 1994). The 20 standard amino acid residues (R) were then successively joined in pairs to both sides of these backbones to structure the amino and carboxyl termini (NH2–R–Phe-Gly-Asn-Pro-Gln-Ser-Thr-Tyr-R–COOH). This unified model contains 20 short-chain polypeptides (4940 atoms in all) that share a common central sequence and sample the 20 different amino acid residues at each end. Second, the highest bridging energy between the Trp-metal and the FQA was determined on the basis of the QM/MM computation. Additionally, the wave functions from the computation were analyzed by the Multiwfn program. Bridging energies for the combination of Trp with each of the eight FQAs via a metal girder were computed based on a frequency analysis from the “FQA–Ca/Mg–Trp residue” model. The calculation was as follows:

Bridging energy \( (\Delta G) = G_{\text{FQA-metal-Trp}} - (G_{\text{FQA-metal}} + G_{\text{Trp}}). \) \( (4) \)

2.7. Bonding type and bridging energy

The wave functions from the computation were analyzed by the Multiwfn program (Lu and Chen, 2012). Many pieces of information were extracted by this software, including the reduced density gradient (RDG), the bridging energy, and the Lorentz oscillator. The Lorentz oscillators were used to predict the FTIR spectra, which were then compared with our experimental results. The bridging site at the Trp residue predicted by the FTIR computation was confirmed with the experimental FTIR, which supported the reliability of the model. Based on the functions of the CIP–metal–Trp residue combination, a topological analysis and a graphic illustration of the distribution of the electron density were also performed using the Multiwfn program (Lu and Chen, 2012). The theoretical method for the discrimination of different bonding types can be explained by the quantum mechanical electron density \( (\rho(r)) \) (Johnson et al., 2010; Kang et al., 2015):

\[
\text{RDG}(r) = \frac{1}{2(3\pi^2)^{1/2}} \frac{|\nabla \rho(r)|}{\rho(r)^{3/2}},
\]

where \( \rho(r) \), \( |\nabla \rho(r)| \), and \( \nabla \) are the quantum mechanical electron density, the gradient operator, and the modular arithmetic of the gradient operator for the quantum mechanical electron density, respectively. The bonding types were identified and drawn with interactive isosurfaces around the metal bridge. In the colored contour planes between atoms, we used different colors to represent the bonding types between the metal and the CIP/residue. Blue, green and, red were used to represent strong coordinate bonds, weak vdW bonds, and intermolecular repulsion, respectively. The bridging energies for the combination of Trp with each of the eight FQAs via a metal girder were computed based on a frequency analysis from the “FQA–Ca/Mg–Trp residue” model. The calculation was as follows:

\[
\text{Bridging energy} = G_{\text{FQA-metal-Trp}} - (G_{\text{FQA-metal}} + G_{\text{Trp}}).
\]

3. Results and discussion

3.1. Interactions of a model FQA, CIP, with biofilms are enhanced by a metal bridge

Fig. 1a–d presents a group of CLSM images that demonstrate the metal-enhanced CIP-biofilm association on the surface of \( E. \) coli. In the absence of alkali–earth metals and CIP, a fluorescence signal was not observed, as is apparent in Fig. 1a. After the addition of only CIP (1.25 \( \mu \)mol/L), some faint and scattered rods are visible in Fig. 1b, indicating a weak association of CIP with \( E. \) coli biofilms. In the presence of the CIP antibiotic (1.25 \( \mu \)mol/L) and alkali–earth \( \text{Ca}^2+ \) (2.0 mmol/L), many bright rods are visible in Fig. 1c, suggesting the \( \text{Ca}^2+ \)-enhanced attachment of CIP molecules to extracellular biofilms. Similarly, evidence from the CIP (1.25 \( \mu \)mol/L) and alkali–earth \( \text{Mg}^2+ \) (2.0 mmol/L) conditions reveals numerous bright rods (Fig. 1d), confirming the \( \text{Mg}^2+ \)-enhanced attachment. These results confirm that alkali–earth metals can enhance the association of CIP with biofilms.

Fig. 1e and f presents the core-level \( \text{Ca}2p \) and \( \text{Mg}2p \) signals from XPS, respectively. In the reference \( \text{CaSO}_4 \) sample, the \( \text{Ca}2p_3/2 \) and
Ca2p1/2 peaks were located at 347.7 and 351.2 eV, respectively. After the addition of Ca2+ to aqueous E. coli, Ca2p1/2 produced two peaks (primary 347.25 eV and secondary 346.7 eV; Fig. 1e) that shifted towards a lower energy by 0.45 and 3.95 eV, respectively. The Ca2p1/2 peak shifted towards a lower energy by 0.1 eV. As CIP (1.25 μmol/L) was subsequently added, the signal continuously shifted towards a lower energy, from a primary peak (Ca2p1/2) of 350.6 eV and a secondary peak of 350.3 eV to lower primary (346.8 eV) and secondary peaks (346.4 eV; shoulder peak). The signal continuously shifted towards a lower energy, indicating that Ca2+ acted as an electron acceptor and attracted redundant electrons from the electron-donating biofilms and CIP (Gosselin et al., 2012; Stipp and Hochella, 1991). Combined with the CLSM result, these findings indicate that the Ca2+−enhanced association of CIP with biofilms on the surface of E. coli occurs through an alkali–earth Ca2+ bridge. Additionally, the Mg2p peak decreased to 50.2 eV when biofilms were added and further decreased to 49.7 eV upon CIP addition in comparison to the reference sample, MgSO4 (51.1 eV) (Fig. 1f). Overall, the XPS peaks of alkaline–earth metals, mediated by biofilms (Fig. 1e and f) and CIP (Fig. S4), shifted towards a lower energy, demonstrating that the CIP−biofilm association is enhanced by alkali–earth metals and is related to the formation of a metal bridge between them.

3.2. Bridging site in biofilms

The CIP−biofilm connection via a metal bridge was analyzed by FTIR spectroscopy (black plots) to further explore the bridging site in biofilms. In the FTIR spectrum of biofilm alone (Fig. 2a), the stretching vibrations of the C=O band at 1675 cm−1 and N−H bending and C−N stretching vibrations at 1600 ± 10 cm−1 are related to amino acid residues or peptides from proteins in extracellular biofilms. The band at 1430 cm−1 denotes the stretching vibration of C−OH in carboxylates (Byler and Susi, 1986; Deacon and Phillips, 1980), whereas the bands near 1115 and 1010 cm−1 are ascribed to the stretching vibrations of hydroxyl and C−O−C in saccharides, respectively (Kang and Zhu, 2013). After the binding of CIP via Ca2+ (Fig. 2b) and Mg2+ bridges (Fig. 2c), the characteristics of the C=O band at 1675 cm−1 became weaker compared with those of biofilms alone (Fig. 2a), suggesting that carbonyl groups in the amino acid residues of extracellular biofilms are responsible for the formation of the metal bridge. The band at 1430 cm−1, which is related to C=O in carboxylates, also became much weaker (Fig. 2b and c). Considering the change in the C=O band at 1675 cm−1, these results suggest that the carboxylates in the amino acid residues of biofilms are responsible for the alkali−earth−metal bridges.

Fig. 2a−c shows a group of computational FTIR spectra (red plots) that were plotted by the Lorentz oscillator (blue plots) based on computation from short polypeptides. After associating Trp carboxyl groups with CIP via Ca2+ (Fig. 2b) or Mg2+ (Fig. 2c), the bands at 1675 and 1430 cm−1 became weaker (decrease in molar absorption coefficients). These results further confirm that carboxyl groups of structured Trp residues may act as the main binding sites associated with the formation of alkali−earth−metal bridges in biofilms (further analysis in Fig. 5). In this study, contribution of COOH in humics to the bridging may be ruled out, because humic and fulvic acids were not observed in the highly sensitive three-dimensional excitation-emission matrix (3DEEM) spectra (Fig. S1), which may be attributed to the lack of long-term humification (Milori et al., 2002).

CIP−biofilm associations via metal bridges may be further analyzed by the 13C NMR spectral fingerprints of biofilms (Fig. 2d). Based on previous work (Wishart et al., 1995), the carboxyl groups in biofilms may be assigned to Trp (176.1 ppm), Tyr (175.8 ppm), Phe (175.7 ppm), Gly (174.9 ppm), Thr (174.7 ppm), Ser (174.4 ppm), Cys (oxidized and reduced states, 174.6 ± 0.2 ppm), and His (174.0 ppm) residues. In Fig. 2d, resonance intensity of Trp residues (176.1 ppm) was dominant in all peaks; a comparison of this signal with different treatments revealed a marked downfield shift (δδ = 0.2) of the resonances attributed to the carbon signals of carboxyl residues in biofilms after treatment of biofilms with Mg/Ca−CIP. The downfield shift indicates that there are some
Fig. 2. FTIR and $^{13}$C NMR spectra of biofilms before and after their association with CIP via alkali–earth Ca$^{2+}$ and Mg$^{2+}$ bridges. FTIR spectra: (a) biofilm alone; (b) biofilm-Ca$^{2+}$-CIP; (c) biofilm-Mg$^{2+}$-CIP. Black plots represent the experimental data. Blue bar charts and red plots represent the Lorentz oscillators and infrared (IR) spectra from the computation at the B3LYP/6-31G(d,p) level with a dispersion-corrected DFT-D3(BJ). Red arrows represent the absorption bands related to the bridging sites. (d) Experimental $^{13}$C NMR spectra from biofilm, biofilm-Ca$^{2+}$-CIP, and biofilm-Mg$^{2+}$-CIP. After the combination of biofilms with Mg/Ca-CIP, the two pink arrows represent two marked downfield shifts ($\Delta \delta = 0.2$) of the resonances that are attributed to the carbon signal of the carboxyl residues in biofilms. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

multinuclear species containing calcium/magnesium ions that are bridged through the carboxylates of Trp (Barszcz et al., 2013), as Trp residues exhibited the highest abundance and the most obvious change in all of the resonance signals.

3.3. Trans-biofilm uptake of FQA

Fig. S2 shows the appearance of E. coli before and after biofilm manipulation, suggesting the removal of biofilms from the surface of low-biofilm E. coli. Fig. 3a shows that the concentration of CIP that was retained in high biofilms (135-nm thick in Fig. S2) increased from $(5.2 \pm 0.3) \times 10^{-3}$ to $2.1 \times 10^{-2}$ mmol/10^9 cells as Ca$^{2+}$ was added at concentrations up to 3.5 mmol/L. However, the concentration of CIP in low biofilms increased only from $(5.2 \pm 0.3) \times 10^{-3}$ to $1.56 \times 10^{-2}$ mmol/10^9 cells. High biofilms on the E. coli surface caused greater retardation of CIP in the extra-cellular matrix. Fig. 3a also shows that, in contrast, the intracellular CIP concentration in high-biofilm E. coli gradually decreased from $(4.0 \pm 0.2) \times 10^{-3}$ to $2.5 \times 10^{-3}$ mmol/10^9 cells when Ca$^{2+}$ was added at concentrations up to 3.5 mmol/L. The intracellular CIP concentration in low-biofilm E. coli decreased to $3.7 \times 10^{-3}$ mmol/10^9 cells with Ca$^{2+}$ addition, but this value was still greater than that in high-biofilm E. coli. These findings confirm that extracellular retardation in biofilms, which is enhanced by alkali–earth–Ca$^{2+}$ bridges, reduces cellular uptake of the CIP antibiotic.

Fig. 3b presents that with the addition of alkali–earth Mg$^{2+}$ (0–3.5 mmol/L), the concentration of CIP that was retained in high biofilms on the surface of E. coli increased from $(5.5 \pm 0.5) \times 10^{-3}$ to $2.9 \times 10^{-2}$ mmol/10^9 cells, whereas the concentration in low biofilms decreased less, from $(5.5 \pm 0.5) \times 10^{-3}$ to $1.8 \times 10^{-2}$ mmol/10^9 cells. Correspondingly, the intracellular CIP concentration in low-biofilm E. coli decreased to a final value of $2.7 \times 10^{-3}$ mmol/10^9 cells, which was slightly higher than that in high-biofilm E. coli ($2.4 \times 10^{-3}$ mmol/10^9 cells). Similar findings reconfirm that high biofilms on bacterial surfaces, which are enhanced by the formation of alkali–earth–metal bridges, restrict the cellular uptake of CIP.

Fig. 3c shows the trans-biofilm transport rate of CIP into cells (r) with the addition of alkali–earth metals. As the Ca$^{2+}$ concentration increased up to 3.5 mmol/L (CIP = 1.25 mmol/L), the r value for high-biofilm E. coli decreased from $(2.06 \pm 0.02) \times 10^{-4}$ to $(1.09 \pm 10^{-4}$ mmol/µm² h 10^9 cells. In the low-biofilm E. coli, the r value was reduced from $(2.06 \pm 0.02) \times 10^{-4}$ to $(1.75 \times 10^{-4}$ mmol/µm² h 10^9 cells. High biofilms act to lower the trans-biofilm transport rate of CIP, and alkali–earth–metal bridges formed in high biofilms will have greater potential for decreasing trans-biofilm antibiotic transport. In the presence of Mg$^{2+}$ (0–3.5 mmol/L), the r value was reduced from $(2.10 \pm 0.01) \times 10^{-4}$ to $(1.31 \times 10^{-4}$ mmol/µm² h 10^9 cells in high-biofilm E. coli and to $(2.10 \pm 0.01) \times 10^{-4}$ mmol/µm² h 10^9 cells in low-biofilm E. coli. This finding further supports the notion that the alkali–earth–metal...
bridges formed in high biofilms are not as conducive to the trans-biofilm transport of CIP. These comparable results regarding extra- and intra-cellular distribution underline an important role for the alkali–earth–metal bridges in reducing the cellular uptake of FQAs.

On the basis of the extra- and intra-cellular distribution in Figs. 3 and 4 depicts the association of E. coli growth with the distribution of CIP. In presence of alkali–earth Ca\(^{2+}\) (0–3.5 mmol/L, initially added CIP = 1.25 \(\mu\)mol/L), the E. coli density was enhanced to 0.42 \(\times\) 10\(^8\) cells/mL for high-biofilm manipulation (retardation: 2.08 \(\times\) 10\(^{-2}\) \(\mu\)mol CIP/10\(^9\) cells; uptake: 2.13 \(\times\) 10\(^{-2}\) \(\mu\)mol CIP/10\(^9\) cells) and to 0.359 \(\times\) 10\(^8\) cells/mL for low-biofilm manipulation (retardation: 1.58 \(\times\) 10\(^{-2}\) \(\mu\)mol CIP/10\(^9\) cells; uptake: 3.49 \(\times\) 10\(^{-2}\) \(\mu\)mol CIP/10\(^9\) cells) (Fig. 4a and b). The highest growth density corresponded to the highest extracellular retardation and the lowest cellular uptake of CIP, which demonstrates that the formation of Ca\(^{2+}\) bridges on the surface of E. coli has a positive effect on the protection of E. coli cells. In the presence of alkali–earth Mg\(^{2+}\) (0–3.5 mmol/L, initially added CIP = 1.25 \(\mu\)mol/L), E. coli densities were enhanced to maximal levels of 0.388 \(\times\) 10\(^8\) cells/mL for high-biofilm E. coli and 0.365 \(\times\) 10\(^8\) cells/mL for low-biofilm E. coli (Fig. 4c and d). Accordingly, the CIP retardation values were 0.392 \(\times\) 10\(^{-2}\) (high biofilms) and 0.365 \(\times\) 10\(^{-2}\) \(\mu\)mol CIP/10\(^9\) cells (low biofilms), which corresponded to uptake values of 2.58 \(\times\) 10\(^{-2}\) and (4.08–4.18) \(\times\) 10\(^{-3}\) \(\mu\)mol CIP/10\(^9\) cells, respectively. The high-biofilm E. coli causes a higher density than the low-biofilm E. coli. These results lead us to conclude that the formation of a metal bridge in biofilms facilitates E. coli growth by enhancing extracellular retardation of CIP.

### 3.4. Bond types at key sites

Fig. 5a presents results for structured short polypeptides on the basis of the quantum mechanical/molecular mechanical (QM/MM) computation. Metal girder-centered local images are presented in Fig. 5b, d, and f. The electronegative carbonyl groups in FQAs interact with the carbonyl groups of Trp residues in the extracellular layer of the CIP antibiotic. These observations were consistent with previously simulated results from molecular docking between NOX and protein (Lu et al., 2010), suggesting that the CIP–biofilm interaction is based primarily on H bonds.

The gradient isosurfaces exhibited a rich visualization of metal electron donor–acceptor (metal EDA) interactions (Fig. 5d). Four planar isosurfaces (blue) indicate a strong attraction...
between Ca\(^{2+}\) and oxygen atoms. The non-bonded interaction between a pair of oxygen atoms in Trp residues, which is also indicated by the red isosurface, shows the repulsion between them. On the CIP side (the left side of Fig. 5d), both of the oxygen atoms in the deprotonated carboxyl group and its adjacent carbonyl in the aromatic nucleus together contacted the Ca\(^{2+}\)-bridge girder through a blue oval-shaped gradient isosurface, indicating a strong n–metal EDA attraction. On the biofilm side (the right side of Fig. 5d), a pair of oxygen atoms in a deprotonated carboxyl group of a tryptophan residue were associated with a Ca\(^{2+}\)-bridge girder through another blue oval-shaped gradient isosurface, further confirming the presence of another strong n–metal EDA interaction. These results suggest that both carbonyl/deprotonated carboxyl groups in FQAs and deprotonated carboxyl groups in Trp residues are collectively responsible for the formation of alkali–earth–metal bridges.

Fig. 5e shows plots of RDG versus electron density multiplied by the sign of the second Hessian eigenvalue (corresponding to Fig. 5d). Three low-gradient spikes, at 0.01–0.04 au, indicated molecular repulsions between a pair of oxygen atoms in the deprotonated carboxyl group of a Trp residue and between the carbon of the carbonyl and its adjacent carbon in the aromatic nucleus of CIP (corresponding to the red color of the gradient isosurfaces in Fig. 5d). The spike at −0.018 au revealed the weak vdw attraction between one of the oxygen atoms in a deprotonated carboxyl group and its adjacent carbonyl in the CIP molecule (corresponding to the green color of the gradient isosurfaces in Fig. 5d). A pair of spikes (−0.03 to −0.06 au) revealed the strong n–metal EDA attractions around the alkali–earth–metal bridge girder.

In addition to the weak vdw attraction in the molecular interiors of CIP and Trp residues, a strong EDA attraction around the central Mg\(^{2+}\) girder was also indicated by the visual gradient isosurfaces (Fig. 5f). In Fig. 5g, an apparent low-gradient spike, which corresponds to the isosurface of an Mg\(^{2+}\) bridge, has a more negative value at −0.057 a.u., suggesting a stronger EDA interaction of electronegative oxygen atoms with Mg\(^{2+}\) than with Ca\(^{2+}\). This quantitative result showing the greater negativity of sign (\(\lambda_2\)) suggests that a stronger EDA interaction via an Mg\(^{2+}\) bridge might be better able to detain CIP in the extracellular matrices.

### 3.5. Dependency on bridging energy

Association constants between FQAs and the biofilm matrix via metals were obtained by a fluorescence micro titration (Fig. 5s). Fig. 6a shows that the growth rate of E. coli increased linearly along with the enhancement of the association constant between FQAs and the biofilm matrix, which implies that the metal bridge-enhanced biofilm–FQA association protects against bacterial apoptosis. The slopes were 0.03 for Ca\(^{2+}\)-enhanced growth and 0.09 for Mg\(^{2+}\)-enhanced growth. The growth rate (the vertical coordinate) followed the following order: CIP > LOM > NOR > LVX = ENX = OFX = PEF = FLE for Ca\(^{2+}\), and CIP = NOR > ENX > LOM > OFX > LVX > PEF = FLE for Mg\(^{2+}\).

Plots of changes in the Gibbs free energy (\(\Delta G\)) computed at a major Trp residue site versus the association constant obtained by fluorescence microtitration (log\(K_a\)) were used to further explain the dependence of the bridging energy on the alkali–earth–metal...
Fig. 5. Models of CIP interactions with Trp residues in biofilms. (a) Model obtained by QM/MM computation of CIP interacting with Trp residues in biofilms via metal bridges. Magnified gradient isosurfaces (b, d, and f) and the corresponding plots of the reduced density gradient versus the electron density multiplied by the sign of the second Hessian eigenvalues (c, e, and g). (b) and (c), CIP-biofilms. (d) and (e), CIP-Ca\(^{2+}\)-biofilms. (f) and (g), CIP-Mg\(^{2+}\)-biofilms. In (b), (d), and (f), the gradient isosurfaces are colored on a blue-green-red scale according to the values of sign (\(\lambda_2\))\(\rho\), which range from -0.08 to 0.08 au. Blue, green, and red indicate the strong attraction of an n-metal EDA interaction, vdW interaction, and strong non-bonded overlap, respectively. Here, the sign of \(\lambda_2\) is used to distinguish bonded (\(\lambda_2 < 0\), such as a π-π bonding) from non-bonded (\(\lambda_2 > 0\)) interactions (a negative value of \(\lambda_2\) represents strong bonding). The lower the negative value of \(\lambda_2\), the stronger the attraction in the region. The O, C, H, N, and F in the molecular structures are represented as red, cyan, white, blue, and brown spheres, respectively. Solvent (water) effects were implicitly taken into consideration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Computation regarding bridging energy indicates that this metal bridge formed in extracellular biofilms acts through a suppressive trans-biofilm transport to protect against bacterial apoptosis. Without this metal bridge, extracellular biofilms exhibit higher permeability, larger antibiotic uptake towards cells, and profoundly defective chemotaxis. Computation regarding bridging energy indicates that this metal bridge is favorable for bacterial survival. This new perspective will enhance the understanding of bacterially antibiotic resistance involved in the field of natural products.

4. Conclusion

More and more attention has been paid to the challenging issue on environmental contamination of antibiotics in recent years. How microbes regulate the trans-biofilm uptake of FQAs is a fundamental question in environmental chemistry and biology. Traditional research thinks basically that bacterially extracellular biofilm matrices are known as passive defense barrier to impede the invasion of hazardous substance, but not know whether FQA attack may be efficiently defused by a metal-reinforced detoxification mechanism occurring at water-biofilm interface. We recently found that metal bridge formed in biofilm matrices anchors the FQAs, and efficiently defuses the FQA attack through this reinforced detoxification mechanism. Here, we present that a deprotonated carboxyl in the tryptophan residues of biofilms acted as a major bridge site, of which one side is a metal and the other is a metal girder jointly connected to the carboxyl and carbonyl of a FQA. Meanwhile, we further demonstrated that a metal bridge formed in extracellular biofilms acts through a suppressive trans-biofilm transport to protect against bacterial apoptosis. This work was supported by the National Science Foundation of China (Grant Nos. 41401543, 41502170), the National Science Foundation for Postdoctoral Scientists of China (Grant No. 201503107), the Fundamental Research Funds for the Central Universities (KJQN201518), and the Special Fund for Agro-Scientific Research in the Public Interest, China (201503107).

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