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# Copper Complex in Poly(vinyl chloride) as a Nitric Oxide-Generating Catalyst for the Control of Nitrifying Bacterial Biofilms

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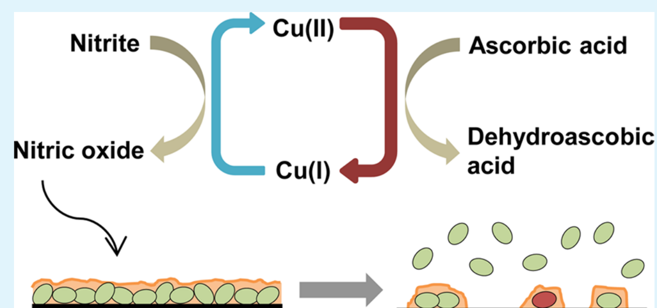
S Supporting Information

**ABSTRACT:** In this study, catalytic generation of nitric oxide by a copper(II) complex embedded within a poly(vinyl chloride) matrix in the presence of nitrite (source of nitric oxide) and ascorbic acid (reducing agent) was shown to effectively control the formation and dispersion of nitrifying bacteria biofilms. Amperometric measurements indicated increased and prolonged generation of nitric oxide with the addition of the copper complex when compared to that with nitrite and ascorbic acid alone. The effectiveness of the copper complex−nitrite−ascorbic acid system for biofilm control was quantified using protein analysis, which showed enhanced biofilm suppression when the copper complex was used in comparison to that with nitrite and ascorbic acid treatment alone. Confocal laser scanning microscopy (CLSM) and LIVE/DEAD staining revealed a reduction in cell surface coverage without a loss of viability with the copper complex and up to 5 mM of nitrite and ascorbic acid, suggesting that the nitric oxide generated from the system inhibits proliferation of the cells on surfaces. Induction of nitric oxide production by the copper complex system also triggered the dispersal of pre-established biofilms. However, the addition of a high concentration of nitrite and ascorbic acid to a pre-established biofilm induced bacterial membrane damage and strongly decreased the metabolic activity of planktonic and biofilm cells, as revealed by CLSM with LIVE/DEAD staining and intracellular adenosine triphosphate measurements, respectively. This study highlights the utility of the catalytic generation of nitric oxide for the long-term suppression and removal of nitrifying bacterial biofilms.




**KEYWORDS:** *biofilm, antibiofilm, nitrifying bacteria, nitric oxide, copper(II)*

## 1. INTRODUCTION

Biofilms, which are communities of bacteria that adhere to each other within a matrix of self-produced extracellular polymeric substances (EPS), represent a key adaptive strategy that confer bacteria with increased resistance to toxins, antimicrobials, and predators. Bacterial biofilms can form virtually on every surface that is in contact with water, and because of their intrinsic resistance, they can cause a range of problems in clinical and industrial settings. For instance, biofilms can form on catheters, prosthetic implants, and tissues, which often cause chronic and persistent infections that antibiotic therapies fail to fully eradicate. In industrial settings, biofilms commonly form on water distribution pipes, causing corrosion, acting as reservoir for potential pathogenic organisms, or accelerating the decay of disinfection agents such as chlorine and chloramine. Biofilms can also clog filtration membranes or foul heat exchanger systems.[1] Conventional treatment methods of killing bacteria through antimicrobial agents and disinfectants are often ineffective at controlling the growth of biofilms on surfaces. Moreover, the use of antimicrobial agents may stimulate the growth of more resistant biofilms, which drives the need for alternative antibiofilm agents, such as nitric oxide.[2,3] Nitric oxide is a free radical gas that functions as an important signaling molecule in many biological systems including biofilms. At low concentrations, nitric oxide has been shown to be very effective at inducing biofilm dispersal through its signaling pathway, resulting in the removal of the biofilm cells in a nontoxic manner.[4,5] At high concentrations, nitric oxide goes through reactions producing reactive nitrogen species that can inhibit the bacterial growth (bacteriostatic) or kill bacteria

Received: March 19, 2015
Accepted: September 29, 2015
Published: September 29, 2015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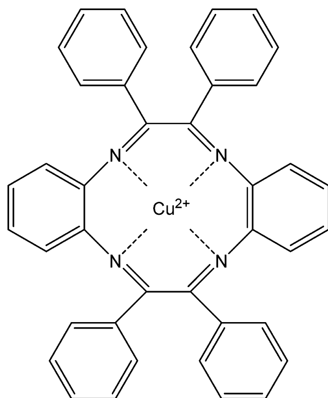
DOI: 10.1021/acsami.5b07971
ACS Appl. Mater. Interfaces 2015, 7, 22148−22156

(bactericidal).[6,7] To date, nitric oxide has not been shown to trigger the formation of more resistant bacteria.[8] Nonetheless, practical use of nitric oxide as an antibiofilm agent is still limited by the need to maintain its sustained release over time. Some studies have concentrated on the use of materials that can store and release nitric oxide, such as polymeric materials,[9−11] zeolites,[12] and metallic and silica nanoparticles.[13−15] However, the nitric oxide-release longevity of such materials is limited by the finite reservoir of nitric oxide gas or donors (such as *N*-diazeniumdiolate and *S*-nitrosothiol)[16] that can be loaded up front. A promising alternative is to use a catalytic technology that allows nitric oxide to be continuously formed in the presence of appropriate reactants. In bacterial systems, the production of nitric oxide from nitrite is completed by nitrite reductase enzymes, with copper(I) species as the active reaction site.[17,18] Indeed, the utilization of copper(I) as an active catalyst for nitrite reduction to nitric oxide has been shown before.[17−19]

Oh and Meyerhoff[18] reported the use of copper(II) or copper(II)/ligand (copper(II) complex) that can be used to generate nitric oxide from a nitrite−ascorbic acid solution. This reaction can occur both in solution and at the interface of hydrophobic polymeric films where a copper(II) complex is immobilized. Moreover, the occurrence of copper species on the polymer surface may offer an additional antimicrobial effect,[20] enhancing its potential as an antibacterial surface. This method also permits the amount and the timing of nitric oxide generation to be controlled by varying the amounts of nitrite and ascorbic acid and the time of their addition, respectively. One potential area where such a system could be readily applicable as a novel biofilm control agent is in cooling tower systems, whereby sodium nitrite is routinely added as a corrosion inhibitor and the acidic nature of ascorbic acid may help to control mineral scale build-up. However, the ability of the catalytic generation of nitric oxide from polymer surfaces containing a copper complex to prevent and remove biofilms has not been proven. In this study, we investigate the use of CuDTTCT (copper(II)-dibenzo[e,k]-2,3,8,9-tetraphenyl-1,4,7,10-tetraaza-cyclododeca-1,3,7,9-tetraene; Scheme 1), which was solvent-cast in poly(vinyl chloride), to generate nitric oxide and examine the effectiveness of the system in suppressing biofilm formation and in biofilm removal on the polymer surface and nearby surface (the wells) where the biofilm was grown. Nitrifying bacteria, an environmentally and industrially relevant chemoautotroph microorganism frequently found in water distribution systems, are chosen as a model organism.

**Scheme 1. CuDTTCT Structure**




## 2. EXPERIMENTAL METHODS

**2.1. Synthesis of Copper Dibenzo[e,k]-2,3,8,9-tetraphenyl-1,4,7,10-tetraaza-cyclododeca-1,3,7,9-tetraene Complex (CuDTTCT Complex).** Dibenzo[e,k]-2,3,8,9-tetraphenyl-1,4,7,10-tetraaza-cyclododeca-1,3,7,9-tetraene (DTTCT) was synthesized following the method described by Oh and Meyerhoff.[18] In brief, 0.05 mol of benzil (Aldrich, 98%) and 0.05 mol of *o*-phenylenediamine (Aldrich, 99.5%) were dissolved in ethanol with a few drops of hydrochloric acid (Ajax Finechem, 32%). The solution was refluxed at 80 °C for 6 h and cooled overnight. On cooling overnight, light brown crystals formed. The precipitated DTTCT crystals were filtered, washed with ethanol (resulting in a color change to a white crystalline product), and dried in a vacuum desiccator. The compound was analyzed by $^1$H NMR (Bruker Advance III 300 MHz). For NMR analysis, 30 mg of dried DTTCT was dissolved in 1 mL of deuterated DMSO.

The CuDTTCT complex was synthesized by refluxing 0.01 mol of DTTCT and 0.05 mol of copper acetate monohydrate (Ajax APS) in ethanol at 80 °C for 2 h. After cooling to room temperature overnight, washing with cold ethanol, and drying in a vacuum desiccator, a light blue precipitate was obtained, indicating the incorporation of copper into the DTTCT structure. For NMR analysis, approximately 10 mg of CuDTTCT was dissolved in 1 mL of deuterated DMSO.

To synthesize CuDTTCT coupon, CuDTTCT powder (2 mg) was dissolved in a premade PVC solution in THF (0.3 mL, 66 mg/mL), and the mixture was sonicated in an ultrasonic bath. The resulting solution was cast onto round glass coverslips (18 mm diameter, ProSciTech). Before use, the glass coverslips were washed with dilute nitric acid, acetone, and ethanol followed by overnight drying at 110 °C. The coupons were dried at 50 °C for 12 h, and the resulting film was removed from the glass coverslips. The amount of Cu loading per coupon was analyzed using inductive coupled plasma optical emission spectrometry (ICP-OES, PerkinElmer Optima 7300). Before analysis, CuDTTCT coupon was weighed and then digested using concentrated nitric acid in a commercially available microwave digestion bomb. From ICP-OES, the amount of copper loading per coupon was found to be 2.88 wt %.

X-ray photoelectron spectroscopy (XPS; ESCALAB250Xi, Thermo Scientific) was used to determine the oxidation state of copper. Prior to XPS analysis, the CuDTTCT coupons were cleaned by brief immersion in toluene to remove any contaminants on the surface. Reduction was performed by immersing the precleaned sample in a 5 mM ascorbic acid solution for 5 min. All samples were dried in a vacuum desiccator for 2 days before analysis.

The extent of leaching of CuDTTCT from the coupon was investigated by incubating the coupon in 2 mL of a nitrifying bacteria media [ATCC medium 2265, which consists of three different stock solutions: stock 1 (final composition in the medium mixture): 25 mM $(NH_4)SO_4$, 3 mM $KH_2PO_4$, 0.7 mM $MgSO_4$, 0.2 mM $CaCl_2$, 0.01 mM $FeSO_4$, 0.02 mM EDTA, 0.5 $\mu$M $CuSO_4$; stock 2: 40 mM $KH_2PO_4$, 4 mM $NaH_2PO_4$, adjusted to pH 8 by 10 M NaOH; stock 3: 4 mM $Na_2CO_3$] for 3 days in the dark (30 °C, 100 rpm). A blank coupon (PVC coupon without the addition of CuDTTCT) was also incubated. At the end of incubation, the supernatants were removed and filtered through a 0.22 $\mu$m membrane. The amount of copper detected in the filtered supernatants was analyzed with ICP-OES (PerkinElmer OPTIMA 7300).

**2.2. Nitric Oxide Generation Measurement.** The amount of nitric oxide that can be generated from CuDTTCT via nitrite reduction was analyzed amperometrically using Apollo TBR4100 free radical analyzer (World Precision Instrument) equipped with an ISO-NOP 2 mm probe. The system was calibrated using *S*-nitroso-*N*-acetylpenicillamine (SNAP; Sigma) and copper sulfate solution according to the manufacturer's protocol. Measurements were performed in sterile nitrifying bacteria media. CuDTTCT coupons or PVC coupons (without any CuDTTCT) as control samples were placed in 20 mL glass vials. Each glass vial was then filled with 10 mL of media and stirred. After a stable baseline was observed, sodium nitrite (Ajax Finechem) and ascorbic acid (Sigma-Aldrich, ≥ 99%) were added into the solution to a final concentration of 5 mM each.


DOI: 10.1021/acsami.5b07971
*ACS Appl. Mater. Interfaces* 2015, 7, 22148−22156

**2.3. Biofilm Suppression Assay.** Biofilm assays were performed in sterile 12-well plates (Corning). A mixed inoculum commercially available nitrifying bacteria for aquarium water purification, comprising *Nitrosospira multiformis*, *Nitrospira marina*, and *Bacillus* sp. (Aquasonic Bio-Culture), was used as a test strain. The mixed inoculum (1 mL) was added into 100 mL of nitrifying bacteria media (ATCC medium 2265) and incubated for 3 days in the dark (30 °C, 100 rpm). Three day old cultures were inoculated into fresh medium, and 2 mL aliquots (OD = 0.008) were added into each well of sterile 12-well plates. Sodium nitrite and ascorbic acid in varying concentrations (ranging from 0.1 to 10 mM) were added into each well. For the experiment where CuDTTCT coupon was used, the CuDTTCT coupon was added into each well, followed by the addition of the sodium nitrite and ascorbic acid solution. The plates were then incubated for 3 days in the dark (30 °C, 100 rpm).

The amount of biomass (both planktonic and biofilm cells) was determined using protein analysis via the bicinchoninic acid method (BCA assay; Sigma). To quantify the growth of planktonic cells, the medium was removed and centrifuged (12 000 rpm, 15 min) to recover the cells. Two washing stages with phosphate-buffered saline (PBS; Oxoid) were used to remove traces of ascorbic acid from the planktonic cells. For biofilm measurements, the wells were also washed twice with PBS to remove traces of ascorbic acid and loosely attached cells. The BCA working reagent (2 mL) was added into each of the wells that contained biofilm and to the centrifuge tubes that contained planktonic bacteria. All wells and tubes were incubated for 30 min (37 °C, 100 rpm), and measurements of the optical density were performed at 562 nm (Infinite M200 Pro, Tecan). The presence of CuDTTCT coupon did not interfere with the assay, as the use of CuDTTCT coupon in the absence of bacteria did not result in a color change of the BCA working reagent, even after the incubation step. Standard solutions using bovine serum albumin, as per the manufacturer's instructions, were also used for each experiment.

Confocal laser scanning microscopy (CLSM) with LIVE/DEAD staining was also performed to quantify the bacteria surface coverage and determine the physiological state (live vs dead) of the adherent bacterial cells. CLSM was performed with an Olympus FluoViewTM FV1000 instrument. Culture aliquots (2 mL each) with OD = 0.008 were grown in 35 mm culture dishes with a coverglass bottom (internal glass diameter 22 mm, ProSciTech) with or without the presence of PVC coupon, CuDTTCT coupon, sodium nitrite, or ascorbic acid and incubated in the dark for 3 days at 30 °C and 100 rpm. At the end of incubation, the medium was removed and the dishes were washed twice with PBS. Adhered cells were stained with 400 $\mu$L of staining solution containing 3.34 $\mu$M SYTO-9 and 19.97 $\mu$M propidium iodide in PBS (LIVE/DEAD *Bac*Light bacterial viability kits L-7007, Molecular Probes Inc.) and incubated at room temperature for a minimum of 15 min. Surface coverage analysis from 12 pictures across the glass bottom was performed on live cells (green channel) using image analysis software (Fiji/ImageJ). All statistical analysis was performed using one-way ANOVA followed by Dunnett's posthoc analysis in Prism (GraphPad).

**2.4. Biofilm Dispersal and Metabolic Activity Assay on Established Biofilms.** Three day old cultures were inoculated into fresh medium, and 2 mL aliquots were added into each well of sterile 12-well plates. A CuDTTCT coupon was added into each well and incubated for 3 days in the dark (30 °C, 100 rpm). One hour before the incubation period ended, sodium nitrite and ascorbic acid solution was added into each well, and incubation continued for 1 h. The amount of biomass was then measured using a BCA assay and CLSM as described before.

Adenosine triphosphate (ATP) analysis (Bactiter Glo, Promega) for biomass metabolic activity measurements was also performed. At the end of a 1 h incubation after the addition of the sodium nitrite and ascorbic acid solution, the planktonic bacteria were collected, centrifuged, washed twice with PBS, and resuspended in 100 $\mu$L of PBS. For biofilm analysis, nitrifying bacteria biofilm samples were washed twice with PBS and resuspended in 2 mL of PBS. To detach the cells, the plate was sonicated for 20 min in a sonicating bath. A 100 $\mu$L sample of the detached cells was mixed with 100 $\mu$L of the Bactiter Glo working reagent and incubated at room temperature for 5 min. The luminescence was measured using a microtiter plate luminometer (Wallac Victor2).

## 3. RESULTS AND DISCUSSION

**3.1. Charaterization of Copper Complex (CuDTTCT) Powder and Coupons.** The DTTCT structure was analyzed using $^{1}$H NMR spectroscopy, and the obtained spectrum (Figure S1) is in accordance with previously reported NMR data.[21] After chelation of the copper(II) ion into the DTTCT complex, $^{1}$H NMR analysis was performed again, and no structural change in the DTTCT complex framework was observed (result not shown).

XPS was used to monitor the oxidation state change of the copper complex before and after the addition of reducing agent (ascorbic acid), and the result is presented in Figure 1. The

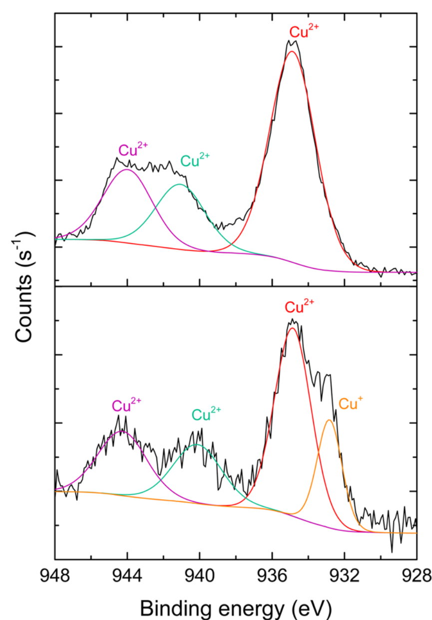



**Figure 1.** XPS spectra of CuDTTCT polymer coupon before (top) and after (bottom) reduction in 5 mM ascorbic acid solution for 5 min.

Cu(II) peak is detected at 934.8 eV, with two Cu(II) shake up peaks detected at 941.0 and 944.0 eV. The reduction of copper(II)-DTTCT to copper(I)-DTTCT upon the addition of ascorbic acid is evident by the emergence of a Cu(I) peak at 932.8 eV (Figure 1, bottom).

The stability of CuDTTCT in PVC upon the addition of nitrite and ascorbic acid was also investigated. Table 1 shows the amount of copper ions that was leached from the coupon during a 3 day incubation in the bacteria medium at different nitrite−ascorbic acid concentrations.

A low level of copper ions can be detected in the absence of CuDTTCT, which originates from the bacteria medium and trace impurities from the nitrite and ascorbic acid reagent. An increase in the amount of copper leached from the CuDTTCT coupon was observed when nitrite and ascorbic acid were added (Table 1). With the addition of 5 mM nitrite and ascorbic acid, the amount of copper leached from CuDTTCT is less than 1%. This suggests an excellent stability of CuDTTCT inside the PVC matrix.

**3.2. Nitric Oxide Generation Measurements.** In order to validate the role of each chemical species in nitric oxide


DOI: 10.1021/acsami.5b07971
*ACS Appl. Mater. Interfaces* 2015, 7, 22148−22156

**Table 1. ICP-OES Measurements of Copper Ions in the Bacteria Medium after Incubation without CuDTTCT (PVC Coupons) and with CuDTTCT Coupons[a]**

| nitrite and ascorbic acid concentration | copper ions (mg/L) PVC coupon | copper ions (mg/L) CuDTTCT coupon | % mass of copper leached out of the CuDTTCT coupon |
|---|---|---|---|
| 0 mM | 0.08 | 1.48 | 0.44% |
| 1 mM | 0.20 | 2.61 | 0.77% |
| 5 mM | 0.32 | 3.29 | 0.94% |

[a]Values shown are the average from duplicate measurements.

generation from the CuDTTCT−nitrite−ascorbic acid mixture, nitrite (5 mM) was first added into the CuDTTCT coupon system, followed by an equimolar amount of 5 mM ascorbic acid. The change in nitric oxide generated was monitored amperometrically. The addition of nitrite led to a small increase in the nitric oxide concentration (Figure 2), whereas

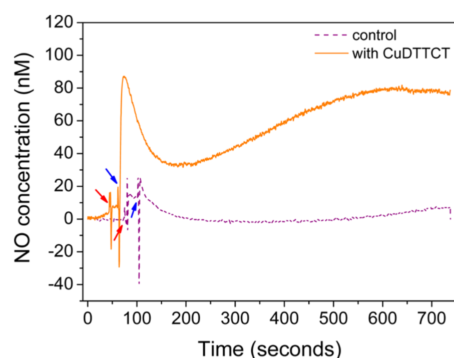
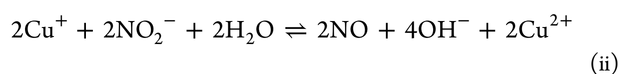



**Figure 2.** Amperometric nitric oxide generation measurement versus time for control (PVC coupon without CuDTTCT) and CuDTTCT coupons (2.88 wt % of copper) in 10 mL of bacteria medium. The red arrow indicates the time at which 5 mM nitrite was added, and the blue arrow indicates the time at which 5 mM ascorbic acid was added.

subsequent addition of ascorbic acid resulted in an abrupt rise in the nitric oxide concentration (up to 80 nM). A similar finding was reported by Oh and Meyerhoff.[18] It was found that nitric oxide is catalytically formed from nitrite via Cu(II)/Cu(I) redox cycling. In detail, ascorbic acid reduces Cu(II) to Cu(I), which reacts with nitrite to produce nitric oxide and, in the process, regenerates Cu(II).[18]

$$2Cu^{2+} + \text{ascorbic acid} \rightleftharpoons 2Cu^{+} + \text{dehydroascorbic acid} + 2H^{+} \quad \text{(i)}$$



$$2Cu^{+} + 2NO_2^{-} + 2H_2O \rightleftharpoons 2NO + 4OH^{-} + 2Cu^{2+} \quad \text{(ii)}$$

The subsequently formed Cu(II) can be reduced again to Cu(I) following the proposed Cu(II)/Cu(I) redox cycling mechanism in the presence of excess ascorbic acid.[18,19] Spectrophotometric measurements show that ascorbic acid can still be detected in the system even after 7 h (Figure S3); therefore, prolonged generation of nitric oxide was observed (Figure 2). Ascorbic acid solution is inherently unstable in the presence of oxygen, which provides strong motivation for investigating the use of other reducing agents that could be used in place of ascorbic acid; this is planned in our future work.

It has been reported that under acidic conditions nitrite solutions can form nitrous acid ($HNO_2$, p$K_a$ of 3.2), which can easily decompose to form nitric oxide and other nitrogen oxides.[22] As the solution pH remained between 7.3 to 7.6 upon the addition of 5 mM nitrite−ascorbic acid, we believe that a major proportion of nitric oxide was generated through the redox cycling pathway rather than through the acidification of nitrite pathway.[22,23] However, we cannot rule out the possible formation of $HNO_2$ in our system, which may contribute to the overall NO release, although the system was well-mixed during the addition of ascorbic acid. A dip in the nitric oxide level was also observed in the period between 100 and 250 s (Figure 2). This can be attributed to the oxidation of the initially generated nitric oxide to nitrite or the oxidation of copper(I) species that decreases the nitric oxide signal momentarily.[18] In the absence of CuDTTCT, a maximum of 20 nM nitric oxide was generated, with no prolonged release observed in an equivalent experimental setup. This confirms the significance of CuDTTCT as a nitric oxide generation catalyst.

### 3.3. Studies on Nitrifying Bacteria Biofilm Suppression.

The effectiveness of nitrite and ascorbic acid solution at reducing biofilm formation was studied first in the absence of CuDTTCT. Here, the presence of biomass was determined by measuring the protein concentration, complemented by confocal laser scanning microscopy (CLSM) of the LIVE/DEAD-stained biomass. The latter analysis accounts for the potential detection of biofilm EPS in the protein assay, which would lead to an overestimation of the number of bacteria on the surface.[24]

In the absence of CuDTTCT, an addition of 5 mM nitrite−ascorbic acid inhibited the growth of biofilm by 40% and of planktonic biomass by 60% relative to the cultures that did not receive any treatment (control, Figure 3a). Approximately 0.32 mg/L of leached copper can be detected from this system (Table 1). However, it is unlikely that the 0.32 mg/L of copper leachate detected caused the observed reduction in biofilm growth, as even 1.5 mg/L of copper ions did not lead to significant biofilm reduction (Figure S4). This suggests that the inhibition effect observed here was due to the nitrite−ascorbic acid mixture. Increasing the amount of nitrite and ascorbic acid to 10 mM led to a severe suppression of the biofilm formation and only 15% planktonic growth as compared to the untreated controls. The trend of the biofilm growth suppression was also observed by using LIVE/DEAD staining in CLSM analysis (Figure 3b). The biofilm surface coverage was reduced by 25% after treatment with 1 mM nitrite and ascorbic acid, which is in close agreement with the ∼20% reduction in biofilm biomass measured with the protein assay after the same treatment. Enhanced reduction in biofilm surface coverage was observed when the nitrite and ascorbic acid concentration was increased to 5 mM, with only ∼30% bacteria detected on the surface when compared to the control. Furthermore, it was shown that the biomass remained viable following the nitrite−ascorbic acid treatment in all samples (Figure 3d,e). It appears that nitrite and ascorbic acid in this case inhibit the cell proliferation of nitrifying bacteria. Such "antibiofilm" activity of the nitrite−ascorbic acid system is in agreement with the earlier reports, in particular on Gram-negative pathogens,[23,25,26] but no study had been done previously on environmentally relevant bacteria, such as nitrifying bacteria. The suppressed biofilm growth in our study appears to result from the quick burst of nitric oxide generation observed in the nitrite−ascorbic acid system (Figure 2).[7,19]



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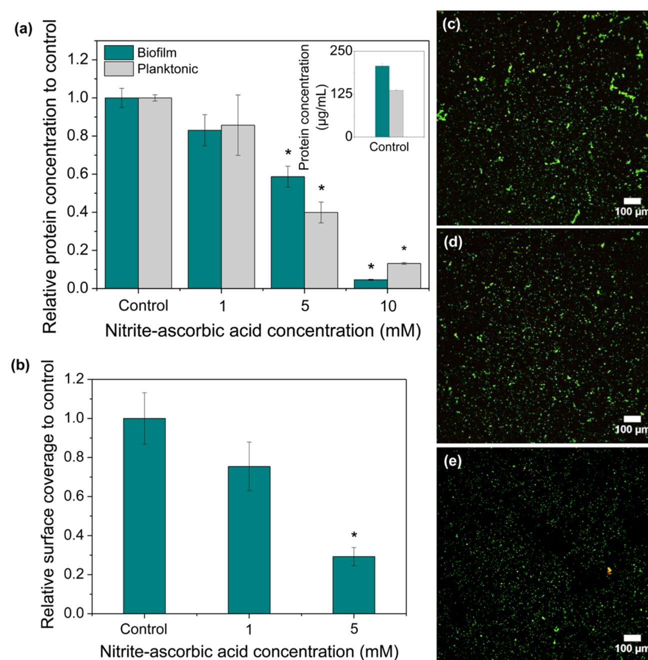


**Figure 3.** (a) Protein measurements of the nitrifying bacteria suppression assay using a nitrite and ascorbic acid mixture. All values shown are normalized to the protein concentration of the control (inset). Error bars indicate standard error between replicates ($n = 3$); $^*p \leq 0.05$ against the control. (b) Surface coverage analysis of the nitrifying bacteria biofilm. Analysis was performed on the green channel of 12 confocal images taken from the bottom of the culture dishes. All values shown are normalized to the surface coverage of the nitrifying bacteria biofilm in medium only (control). Error bars indicate standard error between replicates ($n = 2$); $^*p \leq 0.05$ against the control. Confocal laser scanning microscopy images of nitrifying bacteria biofilm grown (c) in medium only (control) and in the presence of (d) 1 mM and (e) 5 mM nitrite and ascorbic acid. Green stains denote viable bacteria, whereas red and yellow stains denote nonviable bacteria. The copper leachate concentrations detected in control and in the 1 and 5 mM nitrite−ascorbic acid systems were 0.08, 0.20, and 0.32 mg/L, respectively.

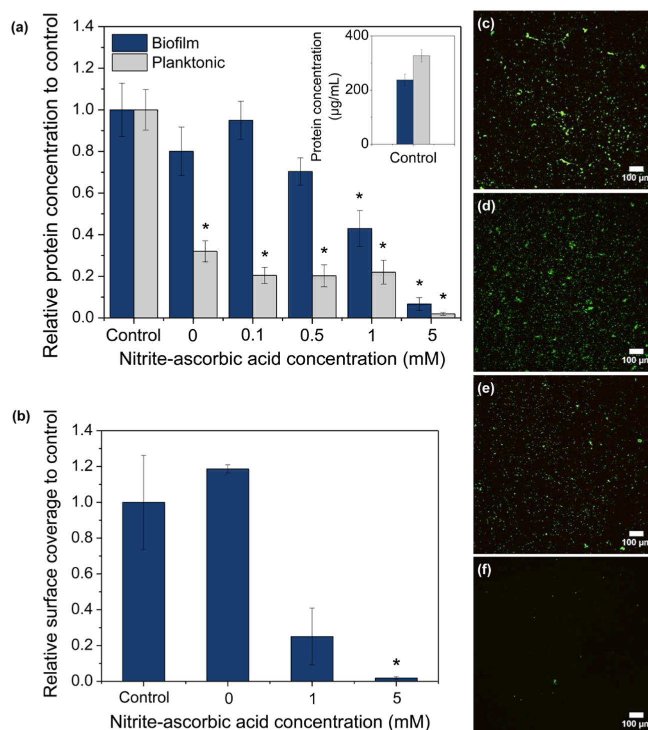


**Figure 4.** (a) Protein measurements of the nitrifying bacteria suppression assay using a CuDTTCT, nitrite, and ascorbic acid mixture. All values shown are normalized to the protein concentration of the control (inset). Error bars indicate standard error between replicates ($n = 4$); $^*p \leq 0.05$ against the control. (b) Surface coverage analysis of a nitrifying bacteria biofilm. Analysis was performed on the green channel of 12 confocal images taken from the bottom of the culture dishes. All values shown are normalized to the surface coverage of the nitrifying bacteria biofilm grown in the presence of PVC coupon (control). Error bars indicate standard error between replicates ($n = 2$); $^*p \leq 0.05$ against the control. Confocal laser scanning microscopy images of nitrifying bacteria biofilm grown in the presence of (c) PVC coupon (control), (d) CuDTTCT coupon, (e) CuDTTCT coupon and 1 mM nitrite−ascorbic acid, and (f) CuDTTCT coupon and 5 mM nitrite−ascorbic acid. Green stain denotes viable bacteria, whereas red and yellow stains denote nonviable bacteria. The copper leachate concentrations detected in the 0, 1, and 5 mM nitrite−ascorbic acid systems were 1.48, 2.61, and 3.29 mg/L, respectively.

Recalling the prolonged and 4-fold higher release of nitric oxide (Figure 2), the antibiofilm activity of the CuDTTCT−nitrite−ascorbic acid system is investigated with protein assay−CLSM analyses in the presence of CuDTTCT coupons. Incorporation of CuDTTCT into the PVC coupon alone reduced the planktonic biomass to 32% of the control culture (Figure 4a). This reduction is thought to be the result of copper toxicity, specifically, the soluble copper ions. In order to simulate the effect of leached copper (1.5 mg/L for CuDTTCT alone, Table 1), copper sulfate at an equivalent concentration was used (Figure S4). In the presence of 1.5 mg/L of copper ions, a 65% decrease of planktonic bacteria compared to the control was observed with no significant effect toward the biofilm, which is in accordance with earlier observation in a CuDTTCT-only system. Copper toxicity to planktonic bacteria is well-documented and may involve several mechanisms such as oxidative stress induced by Fenton reaction, inactivation of Fe−S clusters, or lipid peroxidation.[27,28]

Addition of nitrite−ascorbic acid of up to 1 mM in the presence of CuDTTCT further reduced the planktonic and biofilm biomass to ∼20 and ∼43% of the control value, respectively. Such suppression of biofilm formation was not observed at the same nitrite−ascorbic acid concentration in the absence of CuDTTCT. Further increasing the nitrite−ascorbic acid concentration to 5 mM in a CuDTTCT system resulted in a more than 95% reduction of biofilm biomass compared to the control and complete inhibition of planktonic growth. The addition of 5 mM nitrite−ascorbic acid into the CuDTTCT system was associated with detection of 3.3 mg/L leached copper ions, which did not result in a significant reduction in nitrifying biofilm biomass, although a 77% reduction in planktonic biomass was observed (Figure S4). In fact, the presence of 10 mg/L copper ions was not able to fully eradicate the biofilm (only 50% reduction compared to control), as opposed to drastic biofilm reduction observed in the 5 mM CuDTTCT−nitrite−ascorbic acid system. Here, the system's high inhibition of biofilm formation cannot be attributed solely to the leached copper ions. Therefore, this led us to the conclusion that biofilm suppression is predominantly caused by the CuDTTCT−nitrite−ascorbic acid mixture, most likely due to the activity of catalytically generated nitric oxide as a cell proliferation inhibitor. In agreement with the biofilm inhibition trend that was determined by the protein assay, CLSM analysis


DOI: 10.1021/acsami.5b07971
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revealed 25% biofilm surface coverage with the CuDTTCT system in the presence of 1 mM nitrite−ascorbic acid relative to the control ($p$ = 0.058, Figure 4b). Further increasing the nitrite−ascorbic acid concentration to 5 mM significantly inhibited biofilm formation (surface coverage reduced to 2%).

Comparing the two different systems that have been investigated, a higher amount of nitrite and ascorbic acid was needed to observe comparable biomass suppression in the absence of CuDTTCT. For instance, 5 mM nitrite and ascorbic acid in the absence of CuDTTCT was required to reduce the biofilm surface coverage by ∼70% relative to the control (determined by CLSM, Figure 3b), whereas a lower concentration of nitrite and ascorbic acid (1 mM) was sufficient to acquire a comparable biomass reduction in the presence of CuDTTCT (Figure 4b). Similar to the nitrite−ascorbic acid system, the biomass remained viable following the CuDTTCT−nitrite−ascorbic acid treatment (Figure 4d,e), even at the highest concentration tested (5 mM). This implies that nitric oxide generated in the CuDTTCT−nitrite−ascorbic acid system acts as a cell proliferation inhibitor to the nitrifying bacteria. Nitric oxide can acts as an antimicrobial agent by several mechanisms, in particular, its reactivity toward transition metals. The nitric oxide reactivity toward iron ions has been known to inhibit bacterial respiration,[6] whereas the inactivation of zinc metalloproteins by nitric oxide inhibits DNA replication.[29] The synthesis and repair of DNA are also inhibited by reactions between nitric oxide and tyrosyl radicals, which limits the concentration of the DNA precursor.[29]

In comparison to the nitrite−ascorbic acid system, it would be reasonable at this stage to deduce that the enhanced bacteriostatic activity of the CuDTTCT−nitrite−ascorbic acid system is at least in part due to the 4-fold higher nitric oxide release in the quick burst (first 200 s) along with the subsequent prolonged release of nitric oxide. It was shown in earlier studies that a higher amount of nitric oxide released over short durations is more damaging to Gram-negative bacteria than a prolonged release of lower amounts of nitric oxide.[30] Similar findings were also reported on human fibroblasts, where the reduction in fibroblast numbers exhibited by a copper−nitrite−ascorbic acid system has a higher degree of correlation to the amount of nitric oxide generated in the quick burst (first 200 s) rather than the total amount of nitric oxide over 600 s.[19]

**3.4. Nitrifying Bacteria Biofilm Dispersal Studies.** The effect of nitric oxide on an established biofilm was also investigated by adding nitrite and ascorbic acid to a CuDTTCT system following 3 days of biofilm growth. The term "established biofilm" is used to refer to biofilms that cannot be removed through simple washing steps alone, although biofilm maturity may not have been reached yet. The system was found to be effective at dispersing the established biofilm even with as little as 0.1 mM nitrite−ascorbic acid, which resulted in ∼40% less biofilm compared to the CuDTTCT-only system (Figure 5a). The addition of nitrite−ascorbic acid of up to 1 mM was effective at dispersing the biofilm by 40%; however, when the concentration was increased to 5 mM, the dispersal effect was reduced, with only 22% of biofilm dispersal detected ($p$ value = 0.089 against the CuDTTCT coupon with no nitrite and ascorbic acid added). Confirming the trend of biofilm dispersal determined by the protein assay, surface coverage analysis revealed that 1 mM nitrite−ascorbic acid addition can reduce the biofilm surface coverage by ∼50% as compared to the CuDTTCT-only system with no viability loss observed (Figure 5b,e). This dispersal effect was attributed to

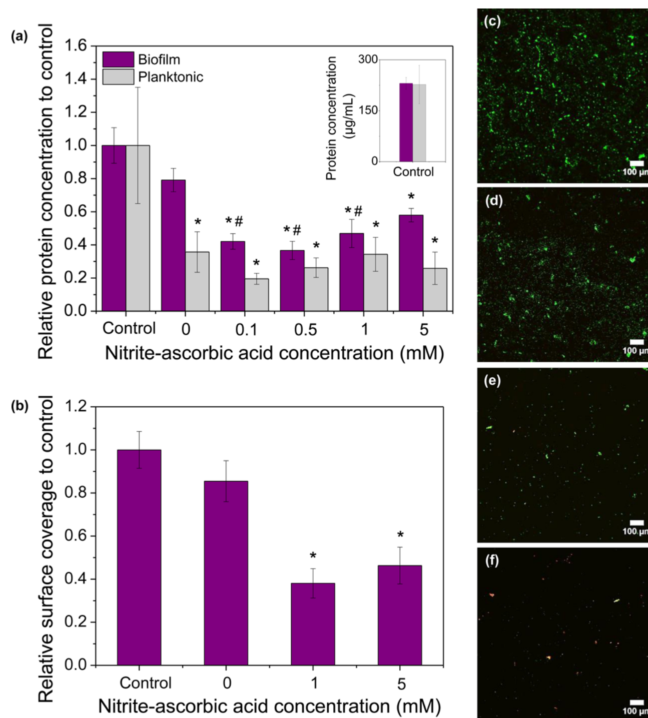


**Figure 5.** (a) Protein measurements of the nitrifying bacteria upon addition of nitrite and ascorbic acid on an established biofilm. All values shown are normalized to the protein concentration of the control (inset). Error bars indicate standard error between replicates ($n$ = 4); $*p \leq 0.05$ against the control (PVC coupon); $\#p \leq 0.05$ against the CuDTTCT coupon. (b) Surface coverage analysis of the nitrifying bacteria biofilm. Analysis was performed on the green channel of 12 confocal images taken from the bottom of the culture dishes. All values shown are normalized to the surface coverage of the nitrifying bacteria biofilm grown in the presence of PVC coupon (control). Error bars indicate standard error between replicates ($n$ = 3); $*p \leq 0.05$ against the control and the CuDTTCT coupon. Confocal laser scanning microscopy images of nitrifying bacteria biofilm grown in the presence of (c) PVC coupon (control), (d) CuDTTCT coupon, (e) CuDTTCT coupon and 1 mM nitrite−ascorbic acid, and (f) CuDTTCT coupon and 5 mM nitrite−ascorbic acid. Nitrite and ascorbic acid were added to an established biofilm grown in the presence of CuDTTCT 1 h before the incubation ended. Green stain denotes viable bacteria, whereas red and yellow stains denote nonviable bacteria. The copper leachate concentrations detected in the 0, 1, and 5 mM nitrite−ascorbic acid systems at the end of final incubation stage were 1.90, 3.34, and 4.54 mg/L, respectively.

nitric oxide generated by the combination of CuDTTCT−nitrite and ascorbic acid. The ability of nitric oxide (by addition of a nitric oxide donor or nitric oxide gas) to cause biofilm dispersal has been reported on a wide spectrum of microorganisms, including Gram-positive *Staphylococcus epidermidis*[5] and *Bacillus licheniformis*,[5] Gram-negative *Pseudomonas aeruginosa*,[4] *Escherichia coli*,[5] *Serratia marcescens*,[5] *Vibrio cholerae*,[5] and *Nitrosomonas europaea*,[31] as well as the *Candida albicans* yeast.[5] In the case of *Nitrosomonas europaea*, which is one of the major members of nitrifying microorganisms in aquatic environments, the switch between biofilm and planktonic states is induced by the regulation of the motility expression and/or chemotaxis responses by nitric oxide, which is potentially the mechanism adopted by the test organism in this study.[31]

From the surface coverage analysis, the addition of 5 mM nitrite and ascorbic acid was also found not to be as effective as

the 1 mM concentration at reducing the biofilm biomass (Figure 5b,e,f). The analysis, however, revealed the presence of dead (red) cells after addition of 5 mM nitrite–ascorbic acid. In this case, it is likely that the cell death occurred at a faster rate relative to the biomass dispersal, thereby reducing the efficacy of biofilm dispersal. The observed toxicity of either the increase in copper leachate concentration or the nitric oxide generated at 5 mM nitrite–ascorbic acid is consistent with the ATP analysis, as described below.

The impact of the CuDTTCT–nitrite–ascorbic acid treatment on the viability of nitrifying bacteria was also assessed by measuring the ATP content of planktonic and biofilm bacteria. The level of ATP detected in biofilms grown in the presence of CuDTTCT was 60% lower than the level of ATP detected in control biofilms (Figure 6). Similarly, planktonic bacteria also had 60% less ATP after exposure to CuDTTCT. Interestingly, although protein and ATP measurements on planktonic bacteria revealed similar trends for control (PVC coupon) and CuDTTCT coupon only, a different trend was observed for the biofilm. While no significant reduction was observed for the biofilm biomass by protein and surface coverage analysis between the control (PVC coupon) and CuDTTCT coupon, 60% lower ATP was detected in biofilms grown in the presence of CuDTTCT coupon compared to control biofilms. This indicates that the presence of CuDTTCT or/and the soluble copper ion could induce stress in the biofilm bacteria, which resulted in a lower amount of ATP being produced rather than removal of the biofilm biomass. A small and insignificant reduction in ATP was observed after the addition of 1 mM nitrite and ascorbic acid ($p$ value = 0.062 for the biofilm and $p$ = 0.074 for the planktonic against the CuDTTCT coupon). However, after the addition of 5 mM nitrite–ascorbic acid, ATP levels were significantly decreased in both biofilm and planktonic bacteria. The decrease in ATP values in this instance could be attributed to viability loss due to cellular damage from nitric oxide exposure as well as the increase in leached copper concentration that is triggered by the addition of nitrite and ascorbic acid. In fact, samples containing CuDTTCT and treated with 5 mM nitrite and ascorbic acid exhibited dead bacteria that were stained with propidium iodide, which is an indicator of cell membrane disruption. Nitric oxide is reported to cause membrane disruption via radical lipid peroxidation.[15] The deterioration of lipid bilayer of the cell membrane could cause the increase in cell surface roughness and membrane degradation.[30] Interestingly, cell death by membrane disruption was not observed when CuDTTCT and 5 mM nitrite and ascorbic acid were added before the biofilm was formed (Figure 4f). It appears that the mode of action from this catalytic system may be dependent on the growth stages of the bacteria. When bacteria are exposed to nitrite, ascorbic acid, and the copper complex from the beginning of incubation, i.e., during the lag phase, bacterial cells may have access to energy resources that allow them to resist potential membrane damage and cell death induced by nitric oxide while being unable to trigger cell division and proliferation mechanisms. However, in established cultures where bacterial cells are in a stationary phase without access to fresh nutrients and have low metabolic activity, the addition of nitrite and ascorbic acid in the presence of copper complex may cause cell death by membrane disruption. Antimicrobial action dependent on the bacterial growth phase has been widely reported before, including for antibiotics.[32] For instance, the polypeptide colistin, which can solubilize the cytoplasmic membrane, was found to be more effective against stationary phase, dormant bacteria with low metabolic activity as compared to cells with high metabolic activity.[33] However, it is also possible that the initial nitric oxide burst generated before the formation of biofilm would induce membrane disruption or reduction of metabolic activity, but the 3 day incubation period allowed some bacterial cells to regain their viability.

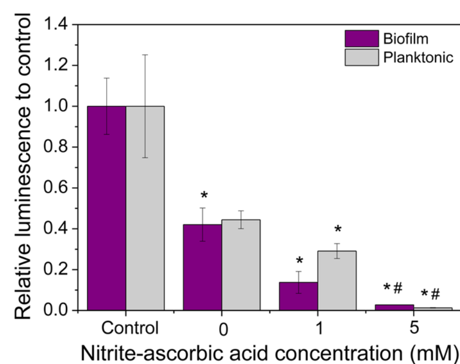


**Figure 6.** Luminescence measurements of the nitrifying bacteria upon the addition of nitrite and ascorbic acid on an established biofilm. All values shown are normalized to the luminescence of the control. Error bars indicate standard error between replicates ($n$ = 2); *$p \leq 0.05$ against the control (PVC coupon); #$p \leq 0.05$ against the CuDTTCT coupon.

## 4. CONCLUSIONS

A lipophilic copper(II) complex (CuDTTCT) as a nitric oxide-generating catalyst has been successfully incorporated into PVC films and tested for the ability to control nitrifying bacteria biofilm formation in the presence of nitrite and ascorbic acid. Copper(II) in the complex was reduced to copper(I) by ascorbic acid, which subsequently reduced nitrite to nitric oxide while being oxidized back to copper(II). Amperometric measurements revealed an initial surge of nitric oxide generated by the copper complex system upon the addition of ascorbic acid followed by a continuous production of nitric oxide. The potential of catalytically generated nitric oxide to control the formation of and disperse a nitrifying bacteria biofilm was demonstrated. Nitrifying bacteria biofilm biomass was suppressed in the presence of CuDTTCT–nitrite–ascorbic acid, which is predominantly caused by nitric oxide. No significant dead cells were observed in the biofilm suppression studies, which indicates that prior to biofilm formation the nitric oxide generated by copper complex–nitrite–ascorbic acid mainly inhibits cell proliferation. Moreover, the copper complex system with 1 mM nitrite and ascorbic acid produced sufficient nitric oxide to effectively disperse established biofilm. At a higher concentration of nitrite and ascorbic acid (5 mM), the biofilm dispersal effect was reduced and significant dead cells were observed. In conclusion, this study highlighted the use of copper complex-bound PVC and nitrite–ascorbic acid to minimize the formation of and to disperse nitrifying bacteria biofilms through sustained generation of nitric oxide. This finding has potential for use in applications where continuous release of an antibiofilm agent is required.


DOI: 10.1021/acsami.5b07971
ACS Appl. Mater. Interfaces 2015, 7, 22148–22156

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b07971.

DTTCT NMR spectrum, immobilization of copper complex in PVC matrix analyzed by SEM-EDS, spectrophotometric measurement of ascorbic acid concentration change over time, and effect of copper ions (in the form of copper sulfate) toward the test organism (PDF).

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This research was supported under Australian Research Council's Linkage Projects funding scheme (LP110100459). We gratefully acknowledge the provision of poly(vinyl chloride) from Chemson Pacific Pty Ltd and financial support from the Australian Water Quality Centre (SA Water) and Water Corporation (Western Australia). We would like to thank Mark Wainwright Analytical Centre for their assistance. We also thank Pejhman Keshvardoust for the development of nitrifying culture system and Doug Lawes for NMR assistance.

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DOI: 10.1021/acsami.5b07971
*ACS Appl. Mater. Interfaces* 2015, 7, 22148−22156