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Ferrous ion as a reducing agent in the generation of antibiofilm nitric oxide from a copper-based catalytic system

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ABSTRACT

The work found that the electron-donating properties of ferrous ions (Fe^{2+}) can be used for the conversion of nitrite (NO_2^-) into the biofilm-dispersing signal nitric oxide (NO) by a copper(II) complex (CuDTTCT) catalyst, a potentially applicable biofilm control technology for the water industries. The availability of Fe^{2+} varied depending on the characteristics of the aqueous systems (phosphate- and carbonate-containing nitrifying bacteria growth medium, NBGM and phosphate buffered saline, PBS at pH 6 to 8, to simulate conditions typically present in the water industries) and was found to affect the production of NO from nitrite by CuDTTCT (casted into PVC). Greater amounts of NO were generated from the CuDTTCT-nitrite-Fe^{2+} systems in PBS compared to those in NBGM, which was associated with the reduced extent of Fe^{2+}-to-Fe^{3+} autoxidation by the iron-precipitating moieties phosphates and carbonate in the former system. Further, acidic conditions at pH 6.0 were found to favor NO production from the catalytic system in both PBS and NBGM compared to neutral or basic pH (pH 7.0 or 8.0). Lower pH was shown to stabilize Fe^{2+} and reduce its autoxidation to Fe^{3+}. These findings will be beneficial for the potential implementation of the NO-generating catalytic technology and indeed, a 'non-killing' biofilm dispersal activity of CuDTTCT-nitrite-Fe^{2+} was observed on nitrifying bacteria biofilms in PBS at pH 6.

1. Introduction

Biofilms are microbial communities that grow within a matrix of extracellular polymeric substances, and are known to cause problems in water industries. In many cases, biofilm formation can decrease heat exchanger or cooling tower efficiency [1]. Biofilm growth in water distribution pipelines could also lead to microbially induced corrosion [2]. Moreover, biofilms have been found to act as a reservoir for the spread of antibiotic resistance genes [3,4]. Conventional methods to control microbial growth using disinfectants such as chlorine and chloramine, are often ineffective at eradicating biofilms due to their increased resistance compared to the free-floating (planktonic) biomass [5]. Therefore, an alternative method for biofilm eradication is required. The utilization of nitric oxide (NO) with its proven efficacy in controlling biofilm formation, appears to be an attractive solution. NO is a free radical gas capable of dispersing biofilms, reverting the biomass to planktonic state *via* a non-toxic pathway [6,7]. Due to its high reactivity and short half-life, research efforts have been dedicated toward the development of sustainable NO generation technologies, capable of delivering NO to the target site [8].

Our previous work has reported the use of a copper(II) complex (copper(II) dibenzo[e,k]-2,3,8,9-tetraphenyl-1,4,7,10-tetraaza-cyclododeca-1,3,7,9-tetraene or CuDTTCT, Scheme 1) that is embedded in a poly(vinyl chloride) (PVC) matrix, to function as a NO generating catalyst [9,10]. The utilization of this copper(II) complex with ascorbic acid as reducing agent generates an active copper(I) species that converts nitrite (typically present in water) to NO [9–12]. The NO-generating catalytic system was able to suppress the growth of nitrifying bacteria, a biofilm-forming consortium commonly found in chloraminated water, as well as dispersing pre-formed nitrifying biofilms [9]. Indeed, our work has also shown the capability of the catalytic system to likely utilize endogenous nitrite (produced by nitrifying bacteria biofilms) for NO production [10]. Yet, the necessity of using an exogenous reducing agent such as ascorbate may not be favorable for

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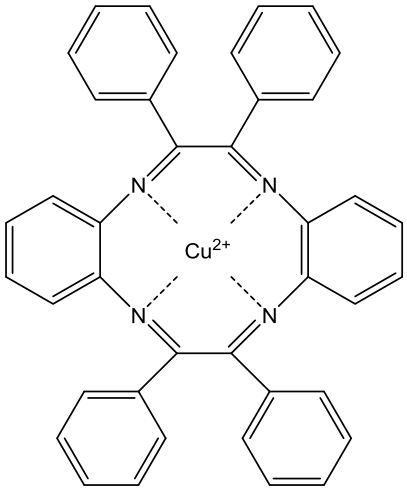

Scheme 1. CuDTTCT Structure.

various applications of this catalytic system in the water industry. In contrast, the possibility of exploiting naturally present chemical moieties (in water) capable of reducing the copper(II) complex catalyst offers a promising alternative. Potential candidates are for instance, humic substances, which have been shown to reduce copper(II) to copper(I) under natural water conditions [13]. Such presence of organic matters however, is generally unwanted in chlorinated/chloraminated water systems due to the likely formation of disinfection by-products. Another inorganic moiety commonly occurring in water systems that holds great potential as a reducing agent is the ferrous ion (Fe^{2+}) [14,15].

Iron usually undergoes chemical (and photochemical) reactions resulting in its rapid cycling between ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms [14,15]. Fe^{2+} ions have been reported to reduce copper(II) species to copper(I) and such interactions could occur either homogeneously (both copper and iron species in solution) or heterogeneously (only copper or iron species in solution) [16]. For instance, oxidation of Fe^{2+} ions in seawater conditions is enhanced by 0.4 log units in the presence of Cu^{2+} ion and, in turn, a strong and rapid reduction of Cu^{2+} to Cu^{+} was observed [17]. In other cases, solid iron in the form of green rusts – a mixed iron(II)/iron(III) hydroxides, have been found to rapidly reduce aqueous Cu^{2+} ion to form solid metallic copper [18]. Contact killing of bacteria on solid iron surface was also observed when it is used in conjunction with Cu^{2+}, which was attributed to the reduction of Cu^{2+} to form the toxic Cu^{+} by the iron surface [19]. In this study, the use of Fe^{2+} (in the form of ferrous chloride) as an alternative reducing agent for the CuDTTCT catalyst (casted into PVC) was investigated in aqueous systems of different characteristics, which contain moieties typically found in the water industries. The pH 6 to 8 aqueous systems contain the iron-precipitating moieties phosphates and carbonate (normally present in water) [20–22] that will affect the Fe^{2+} availability and in turn, the NO-producing capabilities of the catalyst. Ultimately, the effectiveness of the catalytic system in dispersing preformed nitrifying bacteria biofilms was investigated.

2. Material and methods

Synthesis of dibenzo[e,k]-2,3,8,9-tetraphenyl-1,4,7,10-tetraaza-cyclododeca-1,3,7,9-tetraene complex (DTTCT) and its copper complex.

The syntheses of dibenzo[e,k]-2,3,8,9-tetraphenyl-1,4,7,10-tetraaza-cyclododeca-1,3,7,9-tetraene (DTTCT), its copper complex, and the coupon samples were performed following the method described elsewhere [9]. In brief, benzil (0.05 mol; Sigma-Aldrich 98%) and o-phenylenediamine (0.05 mol; Sigma-Aldrich, 99.5%) were refluxed in ethanol with a few drops of hydrochloric acid (Ajax Finechem, 32%) for 6 h at 80 °C. The resultant DTTCT (0.01 mol) was mixed with copper acetate monohydrate (0.05 mol; Ajax APS) in ethanol and refluxed at 80 °C for 6 h.

The coupons were synthesized by mixing the copper complex (2 mg) with pre-dissolved PVC (Chemson Pacific Pty Ltd) in tetrahydrofuran (THF, Chem-Supply) solution (0.3 mL, 66 mg/L) in an ultrasonic bath. Round glass cover slips (18 mm diameter, ProSciTech) were washed with dilute nitric acid, acetone, and ethanol followed by overnight drying at 110 °C before being used as the cast template. A bare coupon (PVC without any copper complex, typical weight ~20 mg) was used as the control. The coupons were dried at 50 °C for 12 h and peeled off from the glass cover slips. Characterizations of the ligand, complex, and coupons have been presented elsewhere [9].

2.1. NO generation measurement

NO generation from the catalytic system was measured amperometrically using an Apollo TBR4100 Free Radical Analyzer (World Precision Instrument) equipped with ISO-NOP 2 mm probe. The system was calibrated using *S*-nitroso-*N*-acetylpenicillamine (SNAP; Sigma-Aldrich) and copper sulfate solution according to manufacturer's protocol. CuDTTCT coupons or bare coupons were placed at the bottom of a 20 mL glass vials equipped with stir bars and filled with 10 mL of testing solution, either the nitrifying bacteria growth medium, the 'NBGM' (ATCC medium 2265 with final pH of 8.0, consisting of three different stock solutions – stock 1 (final composition in the medium mixture): 25 mM $(NH_4)_2SO_4$, 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 μM $CuSO_4$; 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) or PBS (consists of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, prepared using PBS tablet from Sigma-Aldrich). NBGM at pH 6 was prepared by adjusting pH of stock 2 from 4.5 to 5.5 by 10 M NaOH, and mixing it with stock 1 and 3. Concentrated hydrochloric acid was used to adjust the pH of phosphate buffer from 7.4 to 7.0, 6.5, and 6.0. Measurement of the pH was performed using a pH Lab Meter (Metrohm 827, measuring resolution of 0.001 pH) and the pH meter was calibrated before each use. Bare or CuDTTCT coupon was placed at the bottom of the vial and the measurement probe was placed approximately 1 cm from the coupon. Sodium nitrite (Ajax Finechem) and iron(II) chloride tetrahydrate (Sigma Aldrich, ≥99%) was used as the catalytic reactant. At the end of the measurement, an NO scavenger namely 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; Alexis Biochemicals) was added to re-establish the baseline. All measurements were performed in the presence of ambient oxygen.

2.2. Iron speciation analysis

The concentration of Fe^{2+}/Fe^{3+} in the solution was analyzed over time *via* ferrozine spectroscopy method. Colour reagent ferrozine (Aldrich), reducing agent hydroxylamine hydrochloric acid (Sigma-Aldrich), and ammonium acetate buffer (Ajax) were prepared as described by Viollier etc [20]. Two mL of sample solution was added into 12 well plates (Corning) containing either PVC coupon or CuDTTCT coupon, followed by addition of equimolar amounts of nitrite and Fe^{2+}. Iron speciation measurements were performed for 2 h, with two 100 μL aliquots of the sample collected every 3 min or 20 min. The first aliquots were treated with ferrozine and represent the concentration of Fe^{2+} while the second aliquots were treated with ferrozine, reducing agent and buffer to depict the concentration of total iron. The absorbance of the Fe^{2+}–ferrozine complex after incubation of 5 min in room temperature was measured using UV–Vis spectrophotometer (Varian Cary 300) at 562 nm.