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In Situ Reactivity of Electrochemically Generated Nitro Radical Anion on Tinidazole and Its Monomeric and Dimeric Cu^{II} Complexes on Model Biological Targets with Relative Manifestation of Preventing Bacterial Biofilm Formation

Promita Nandy, Ramesh C. Santra, Dibyajit Lahiri, Moupriya Nag, and Saurabh Das*

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ABSTRACT: Formation of nitro radical anion ($-NO_2^{\bullet-}$) and other reduction products of 5-nitroimidazoles, although important for antimicrobial activity, makes the drugs neurotoxic. Hence, an appropriate generation and their role in the free radical pathway needs proper realization. This was attempted by studying the action of tinidazole and its Cu^{II} complexes on model targets (nucleic acid bases and calf thymus DNA). Results obtained were correlated with studies on biological species where prevention of biofilm formation on *Staphylococcus aureus* and *Pseudomonas aeruginosa* was followed. Tinidazole and its Cu^{II} complexes subjected to electrochemical reduction in aqueous solution, under de-aerated conditions, interact with model nucleic acid bases and calf thymus DNA. These model targets were followed to realize what happens when such compounds undergo enzymatic reduction within cells of microorganisms that they eventually kill. Studies reveal that Cu^{II} complexes were better in modifying nucleic acid bases and calf thymus DNA than tinidazole; damage caused to nucleic acid bases was correlated with that caused to DNA, indicating that compounds affect DNA rich in thymine and adenine. Minimum bactericidal concentrations on sessile *S. aureus* and *P. aeruginosa* for the monomeric Cu^{II} complex were 12.5 and 20.25 μM respectively, while those for the dimeric complex were 40.0 and 45.0 μM, respectively. Biofilm formation by *P. aeruginosa* and *S. aureus* and viability count of sessile cells were also determined. Cu^{II} complexes of tinidazole brought about substantial reduction in carbohydrate and protein content in *S. aureus* and *P. aeruginosa*. Downregulation of quorum sensing signaling mechanism viz. reduced production of pyocyanin and elastase during biofilm formation was also detected. Cu^{II} complexes showed much higher tendency to prevent biofilm formation than tinidazole, almost comparable to amoxicillin, an established drug in this regard.
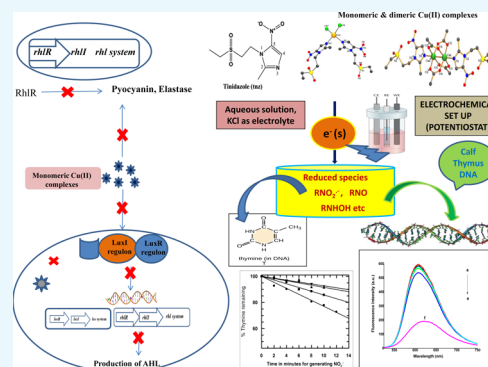
INTRODUCTION

5-Nitroimidazoles are important molecules for pharmaceutical applications and are found in different formulations.[1−5] They address a wide spectrum of medical issues ranging from infections caused by different microbes to being used as anticancer agents in radiotherapy.[1−8] Although metronidazole is the most used, issues like drug resistance and neurotoxicity have resulted in a search for compounds having comparable efficacy but with significantly less adverse effects.[1−3,5−10] Tinidazole (tnz) is a compound that nicely fits this requirement, although conflicting reports on its efficacy and adverse effects do exist.[11−14] Since its antimicrobial activity was first reported, tnz showed a steady increase in acceptability as a drug.[15−18] However, as is true for all 5-nitroimidazoles, its efficacy is accompanied by toxic side effects, which although quoted to be less than metronidazole, do exist.[11−18] The problem with this family of drugs is that, the nitro-radical anion, $RNO_2^{\bullet-}$ (where R represents the portion other than the nitro group) is responsible both for efficacy and toxic side

effects.[11−18] Hence, an approach that enables controlling the generation of $RNO_2^{\bullet-}$ is an extremely relevant issue.[19−21] Within biological systems, 5-nitroimidazoles are first reduced by enzymes pyruvate ferredoxin oxidoreductase[9,22,23] that prepares them for entry into the cells of the target organism. Thereafter, the nitro-radical anion imparts its drug action.

Although related literature mentions $RNO_2^{\bullet-}$ to be responsible for drug action, very few studies have gone into details of such claims that would help us realize their contribution toward cytotoxic action. Research has revealed that complex formation of 5-nitroimidazoles modulate the generation of $RNO_2^{\bullet-}$ that might then be expected to decrease
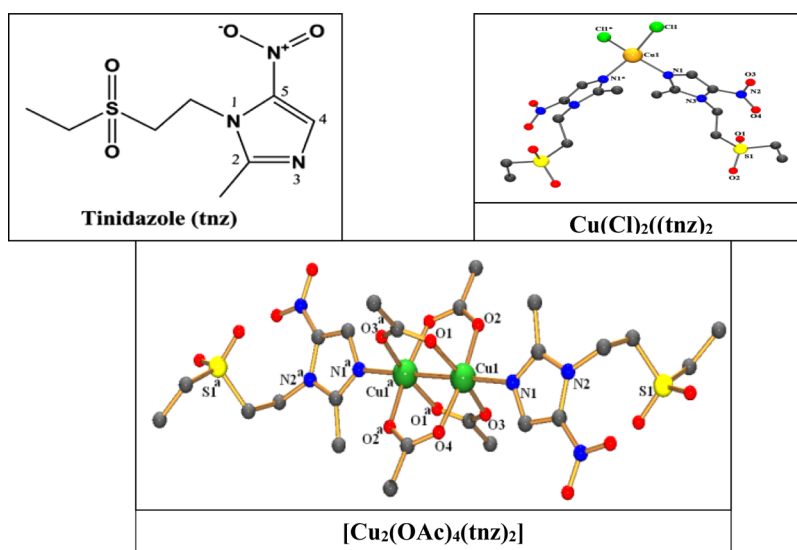
Figure 1. Structure of tnz and its monomeric and dimeric Cu[II] complexes.
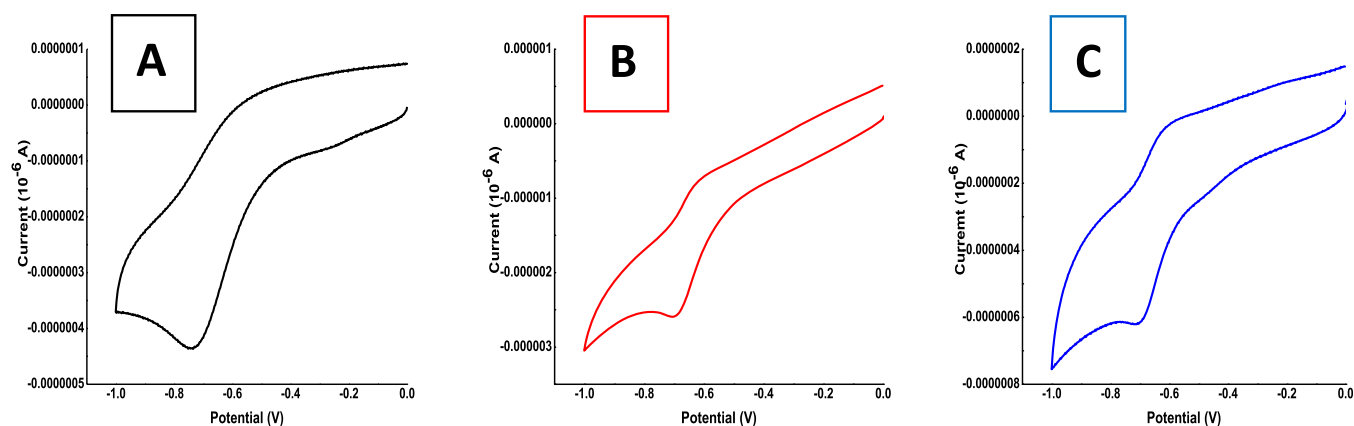

Figure 2. Cyclic voltammograms of 100 μM (A) tnz, (B) its monomeric Cu(II) complex, and (C) its dimeric Cu(II) complex showing a single-step four electron reduction of the nitro group in aqueous solution using a glassy carbon electrode; [NaCl] = 120 mM; scan rate = 0.025 V/s; Ag/AgCl, satd. KCl was used as the reference electrode; and temperature = 303 K.

toxic side effects.[20,21] Because $RNO_2^{\bullet-}$ is important for drug efficacy, its decrease, following complex formation, should logically affect drug action. However, we found from several previous studies that complex formation did not interfere with drug efficacy. In fact, most complexes were either similar in performance on a chosen microbial target when compared with 5-nitroimidazole, from which the complex was prepared, or that the complex performs better.[20,21,24,25] Because complexes result in a decrease in $RNO_2^{\bullet-}$ and yet there is no loss in efficacy, this suggests they have other attributes[20,21,24,25] that enable them to overcome any deficiency that might occur in the free radical pathway.

Through this study, we tried to look at aspects related to cytotoxicity that are either initiated by $RNO_2^{\bullet-}$ or other reduction products formed on the monomeric and dimeric complexes of Cu[II] with tnz (Figure 1) on nucleic acid bases and calf thymus DNA to correlate what might happen when such compounds are enzymatically reduced in biological systems, generating species having the potential to kill disease-causing microbes.[9,20–25]

Reduction products of each compound was generated electrochemically maintaining a glassy carbon electrode at its cathodic peak potential using a method described earlier.[26,27]

In the immediate vicinity of electrochemically generated reduction products, nucleic acid bases or calf thymus DNA were maintained, one at a time so that reduced products react instantaneously. Although the reaction on a model target under laboratory conditions can never be identical with what happens inside cells, it can however shed some light on such processes because similar species were generated enzymatically within cells following the transfer of electron(s) to 5-nitroimidazoles by electron-donating groups present within cells.[9,22,23,28] In situ reactivity of reduction products with nucleic acid bases or calf thymus DNA were subsequently analyzed to realize changes brought about on a target maintained in the immediate vicinity of such generation. To check for a correlation between model studies and the potency of the monomeric and dimeric complexes to inhibit biofilm formation, detailed studies were performed on *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Most bacterial species possess an ability to live in complex sessile communities called biofilm under environmentally stressed conditions. Such sessile micro-colonies remain embedded within self-secreted extracellular polymeric substances (EPSs) and are responsible for the development of major types of nosocomial infections following biofilm
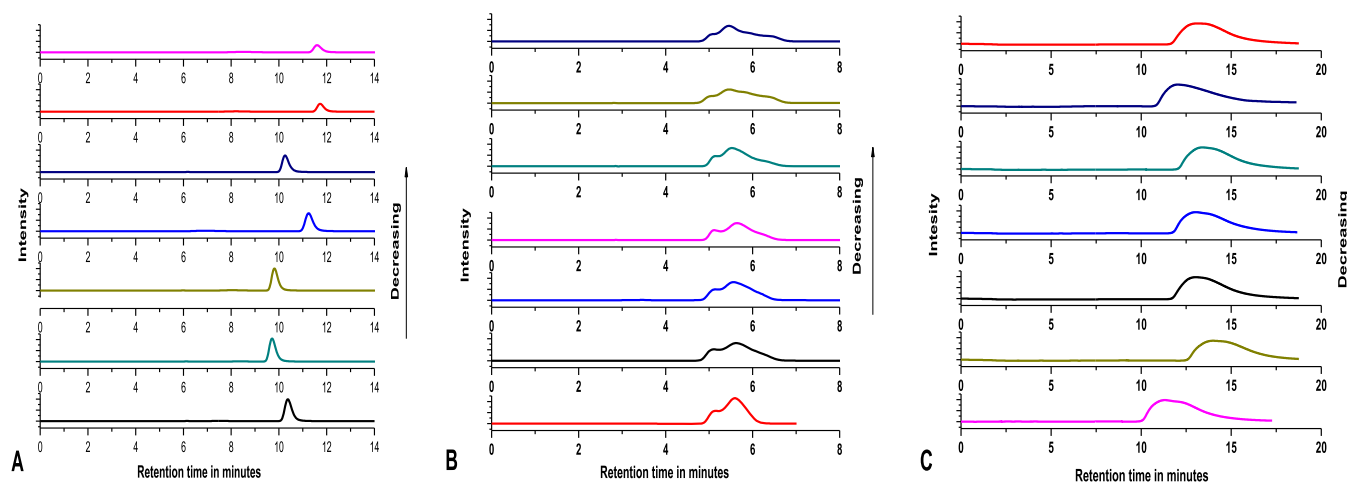
Figure 3. HPLC chromatograms of 10^{-3} M (A) thymine, (B) cytosine, and (C) adenine solutions recorded at 254 nm following them being kept in the immediate vicinity of electrochemically reduced 10^{-4} M $Cu_2^{II}(OAc)_4(tnz)_2$.
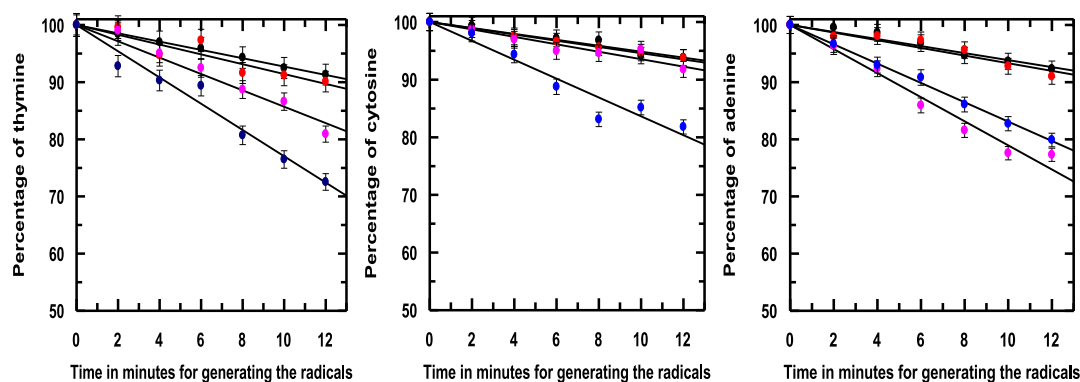

Figure 4. Damage of nucleic acid bases was monitored using HPLC at 254 nm following an electrochemical reduction of compounds in whose immediate vicinity nucleic acid bases were maintained. Respective reduction potentials were −0.745 V for tnz, −0.700 V for the monomeric Cu(II) complex, and −0.710 V for the dimeric Cu(II) complex. Electrochemical reduction was carried out under argon saturated conditions. (Black ●) indicates control experiments when a nucleic acid base was subjected to reduction in the absence of any compound; (red ●) in presence of tnz; (brown ●) in presence of the monomeric complex; and (green ●) in presence of the dimeric complex. [thymine] = [cytosine] = [adenine] = 1×10^{-3} mol dm^{-3}; [tnz] = [monomeric Cu(II)−tnz] = [dimeric Cu(II)−tnz] = 1×10^{-4} mol dm^{-3}.

formation.[29,30] Biofilms are highly resistant both to specific (adaptive) and nonspecific (innate) host defense mechanisms. The development of EPS and subsequent slower diffusion of antimicrobials through the biofilm matrix reduced the rate of metabolism, and so forth, and make bacterial cells less susceptible to phagocytic activities of macrophages and more resistant to antibiotics.[31,32] Such enhancement of resistance resulted in a search for alternate therapies for treating biofilm-associated chronic infections caused by *P. aeruginosa* and *S. aureus*.[33,34] Through this work, we aim to show the potent efficacy of monomeric and dimeric complexes of Cu(II) with tnz (Figure 1) in removing persistent microbial cells of *P. aeruginosa* and *S. aureus*.

■ RESULTS

In Situ Reactivity of Electrochemically Generated Reduction Products. Figure 2 shows voltammograms for tnz, monomeric $[Cu(tnz)_2Cl_2]$, and dimeric $[Cu_2^{II}(OAc)_4(tnz)_2]$ complexes when each was subjected to cyclic voltammetry in an aqueous solution. From the voltammograms, reduction peak potentials of tnz, $[Cu(tnz)_2Cl_2]$, and $[Cu_2^{II}(OAc)_4(tnz)_2]$ were identified at −0.745, −0.700, and −0.710 V, respectively. It may be mentioned here that for 5-nitroimidazoles in

aqueous solution, reduction to the nitro-radical anion is not identified separately; instead, there is a single-step four electron reduction (eq 1).[35,36]

$$RNO_2 + 4e + 4H^+ \rightarrow RNHOH + H_2O \qquad (1)$$

Identification of the potential for the reduction of each compound is important for the study to be undertaken because each compound would have to be reduced to generate suitable reduced species that might interact with a target. Therefore, when in the immediate vicinity of any of the compounds, subjected to electrochemical reduction at a constant potential, nucleic acid bases or calf thymus DNA were maintained, and reduced products would have a high probability to interact with them. Outcome of such interactions was ascertained for nucleic acid bases using HPLC (high-performance liquid chromatography) and for DNA using the ethidium bromide fluorescence technique.[26,27,37]

Interaction of Electrochemically Generated Reduction Products with Nucleic Acid Bases. Various electrochemically reduced species generated in aqueous solution for a compound, following maintenance of a glassy carbon electrode at its cathodic peak potential, for different periods of time,
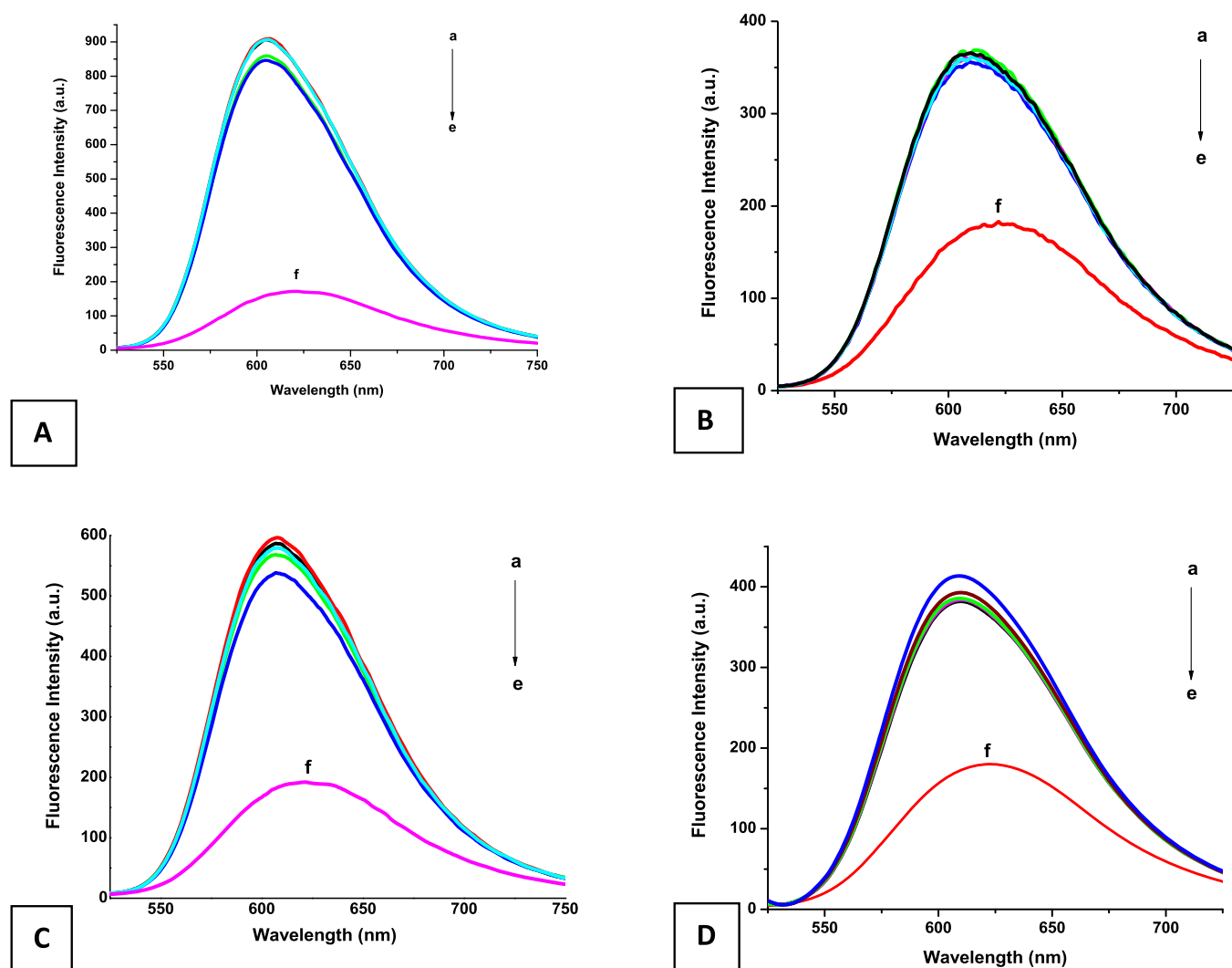
Figure 5. Decrease in fluorescence intensity of the DNA-EtBr adduct recorded at 600 nm (λ_{ex} = 510 nm) following the interaction with electrochemically generated reduced species in (A) absence of any compound, (B) presence of tnz, (c) presence of Cu(tnz)$_2$Cl$_2$, and (D) presence of Cu$_2$(OAc)$_4$(tnz)$_2$ at different time intervals of (i) 0 min, (ii) 5 min, (iv) 10 min, (v) 15 min, and (vi) 20 min. Spectrum (f) in each plot is that of free EtBr.

indicate that it leads to a gradual degradation of nucleic acid bases (Figure 3).

Responses for nucleic acid bases shown in Figure 3 is based on their individual elution peaks under a specific solvent composition eluting them, which was considered as the standard HPLC chromatogram for that nucleic acid base (Figure S1, Supporting Information). Based on elution peaks of individual compounds, degradation plots were quantified (Figure 4). Such standard curves enabled determination of the concentration of nucleic acid bases in the performed experiments. The amount of a nucleic acid base remaining following interaction with reduced species was realized by collecting aliquots from the reaction vessel at different time intervals and evaluating them based on Figure S1, Supporting Information. Figure 4 shows the degradation of nucleic acid bases followed by HPLC at 254 nm after they were allowed to interact with reduced products obtained from tnz and its monomeric and dimeric complexes.[37]

We wanted to generate the data for guanine also because guanine is easily damaged by various radical species. However, owing to issues concerning its solubility in aqueous solution

which despite our best efforts, we had to refrain from going ahead with it. The results we got with guanine were erratic and inconsistent. Hence, we decided to discuss the data obtained for thymine, cytosine, and adenine only. However, if the damage on guanine could be shown, we would have been in a better position to explain the targeting of 5-nitroimidazoles and their metal complexes based on nucleotide content of DNA of the target organism which could then provide a good correlation between an actual drug action reported and this study (Table S1, Supporting Information). Although without guanine this may still be realized, a data for guanine would have made it more convincing.

Interaction of Electrochemically Generated Reduction Products with Calf Thymus DNA. A similar study as the one described above was performed maintaining calf thymus DNA in the immediate vicinity of electrochemically generated reduced species in aqueous solution at pH 7.4 using the same glassy carbon electrode maintained at the identified reduction potential of the compound. In experiments with calf thymus DNA, we subjected the system to slightly longer times than was used for electrochemical reduction of the compounds

to reduced species in case of nucleic acid bases, so that reduced products were produced in greater quantity and there occured a detectable change in DNA, monitored by the fluorescence technique using EtBr.[38−42]

Figure 5 depicts plots showing fluorescence of calf thymus DNA with EtBr, after it was allowed to interact with reduced products generated electrochemically on each compound, in whose vicinity calf thymus DNA was maintained. In each experiment, mixtures of DNA and EtBr were excited at 510 nm and emission was measured at 600 nm.

Modification of calf thymus DNA was realized by plotting percentage of DNA remaining intact against time provided for the generation of electrochemically reduced species on each compound following maintenance of a glassy carbon electrode at the predetermined reduction potential of a compound in an aqueous solution at pH 7.4 (Figure 6). Both Figure 6 and
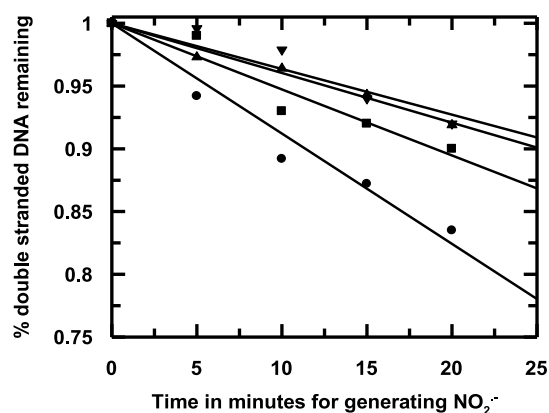

Figure 6. Degradation curves show modification of calf thymus DNA in the absence and presence of either tnz or (▲) its monomeric (■), dimeric (●) Cu(II) complexes; [tnz] = [Cu(tnz)$_2$Cl$_2$] = [Cu$_2$(OAc)$_4$(tnz)$_2$] = 1 × 10^{-4} mol dm^{-3}. (▼) indicates modification of calf thymus DNA in the absence of any compound when subjected to a constant potential of −0.700 V for the same duration of time.

Table 2 indicate the damage caused to calf thymus DNA in the presence of the compounds used in the study. Considering I_0 as the intensity of fluorescence of pure DNA treated with EtBr, I_{EtBr} as intensity of fluorescence for EtBr itself, and I_{expt} as the

intensity of fluorescence of a DNA sample subjected to conditions of the experiment and then treated with EtBr, the fraction of DNA remaining intact is obtained from

$$\text{fraction of DNA remaining} = \frac{(I_{expt.} - I_{EtBr})}{(I_0 - I_{EtBr})}$$

Inhibitory Action of Complexes on Biofilm Formation. *Determination of Minimum Bactericidal Concentration.* The monomeric Cu^{II} complex showed the inhibition of biofilm formation for *S. aureus* and *P. aeruginosa* at concentrations of 12.5 and 20.25 μM, respectively, while that for the dimeric complex was 40 and 45 μM, respectively, suggesting that the monomeric complex showed better efficacy against biofilm formation by cells of *P. aeruginosa* and *S. aureus*. The minimum bactericidal concentrations of tnz for *S. aureus* and *P. aeruginosa* were 50 and 59.25 μM, respectively. Although tnz is an established antibacterial drug,[43,44] very few literature show its antibiofilm properties.[45,46]

Inhibition of Biofilm Formed by *P. aeruginosa* and *S. aureus*. The monomeric complex of Cu^{II} inhibited biofilm formation due to *P. aeruginosa* by 88.52 ± 3.45%, whereas the dimeric complex could decrease it by 76.95 ± 2.29% (amoxicillin reduces biofilm formation by 62.12 ± 2.25%). For *S. aureus* decrease in biofilm formation due to the monomeric complex was by 92.16 ± 4.87%, while for the dimeric complex, it was 81.25 ± 3.55 (amoxicillin decreases it by 72.56 ± 1.29) (monomer *p* < 0.01, dimer *p* < 0.05) (Figure 7).

Disintegration of Structural Component of EPS. EPS matrix of a biofilm comprises a rich supply of nutrients in addition to lipid molecules, nucleic acids, proteins, extracellular DNA, quorum sensing (QS) signaling molecules and water. Hence, removal of biofilm involves strategies that target the EPS matrix leading to its disintegration via a decrease in the synthesis of biomolecules.

The monomeric complex inhibited carbohydrate content within the EPS of the biofilm formed due to *P. aeruginosa* by 75.26 ± 5.8%, the dimeric complex by 71.23 ± 3.55% and amoxicillin by 61.78 ± 2.47%. In case of *S. aureus* for the monomeric complex, the decrease was by 80.29 ± 5.8%, for the dimeric complex, by 75.89 ± 4.7% and for amoxicillin, by 69.56 ± 3.25% (*p* < 0.01). It was further observed that the

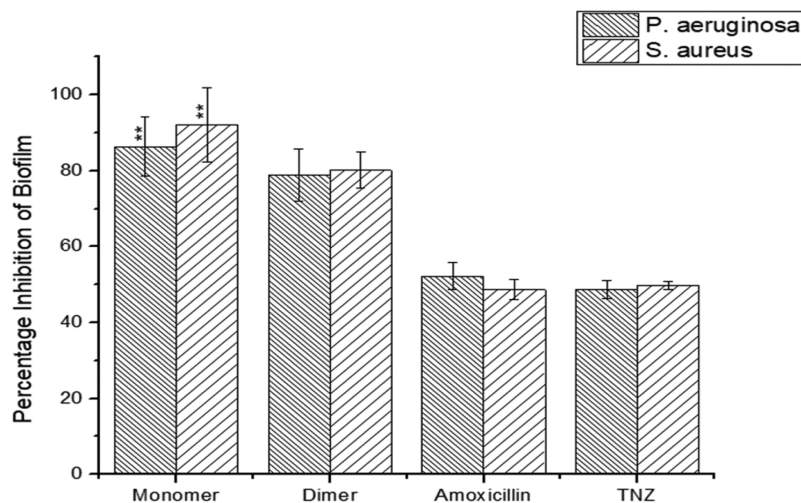**Figure 7.** Maximum inhibition of biofilm formation due to *P. aeruginosa* and *S. aureus* was due to the monomeric complex (*p* < 0.01).
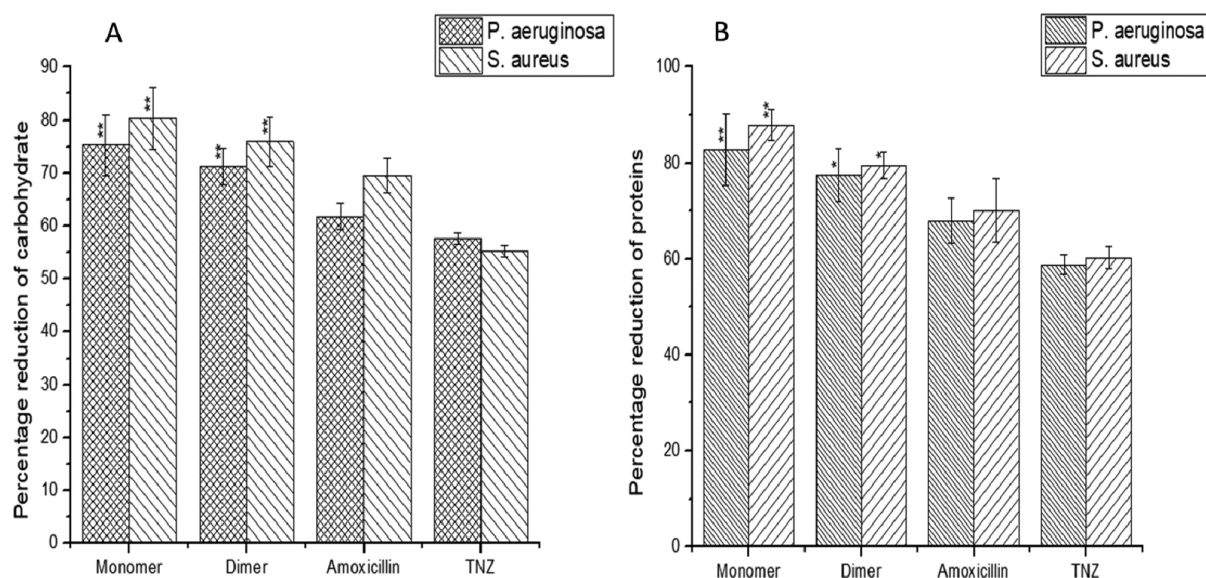
Figure 8. Reduction in carbohydrate (A) and protein (B) present within the EPS.
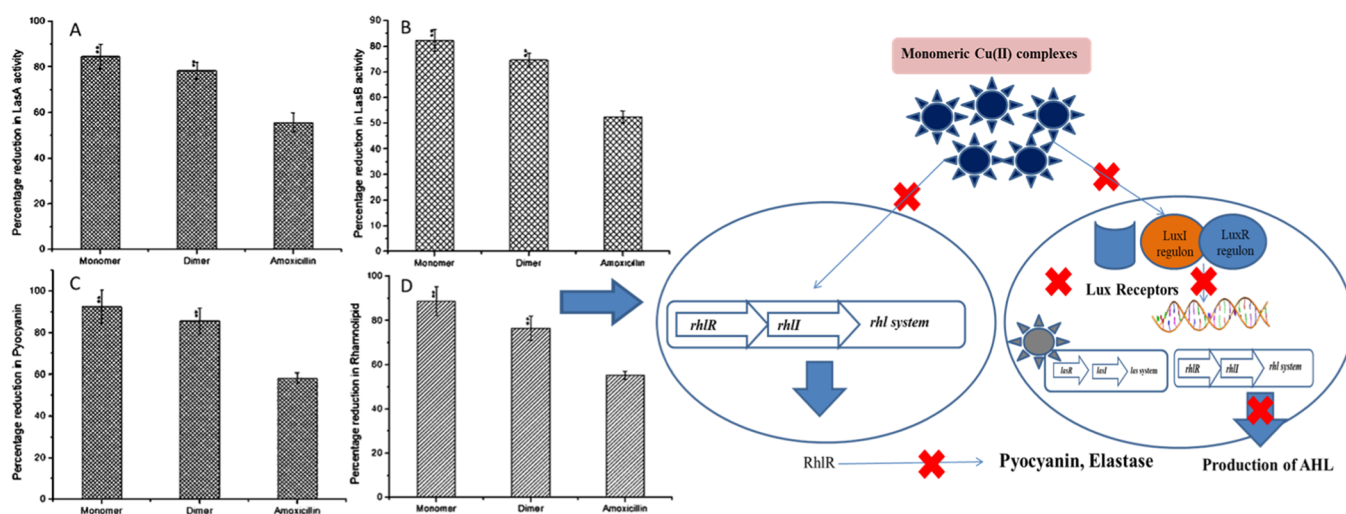

Figure 9. Inhibition of the QS signaling mechanism during biofilm formation.

monomeric complex was able to maximally reduce the protein content of EPS of *P. aeruginosa* and *S. aureus* by 75.26 ± 5.8 and 80.29 ± 5.8%, respectively ($p < 0.01$), which was even higher than that achieved with the standard antibiotic amoxicillin (Figure 8).

Downregulation of the QS Pathway during Biofilm Formation. Antimicrobial potential of monomeric and dimeric complexes of Cu[II] with tnz identifies them as important therapeutic agents. It was earlier observed that the monomeric complex plays a key role in controlling infections caused by microbes.[21] *P. aeruginosa* is known to have many virulence genes viz *LasI/Rhl* that are activated during the QS network leading to the expression of virulence factors such as elastase, rhamnolipid, and pyocyanin.[47] The amount of las A protease and las B elastase was monitored with or without Cu[II] complexes (Figure 9). We observed that *las*-regulated virulence genes *las A* and *las B* were significantly downregulated to 82.4 ± 4.25% in the presence of the monomeric complex (Figure 8 A,B) as compared to the dimeric one or even in comparison to amoxicillin suggesting that the monomeric complex has the ability to block the synthesis of signaling molecules responsible

for regulating biofilm formation by inhibiting LasI/Rhl I synthase.[48] A lack of production of virulence factor pyocyanin after treatment of *P. aeruginosa* with both complexes was observed with a maximum reduction of 86.34 ± 7.25% in the presence of the monomeric complex. Thus experimental results show that the monomeric complex was able to bring about inhibition of QS maximally in *P. aeruginosa*.

DISCUSSION

Maintenance of a glassy carbon electrode at the cathodic peak potential of a compound, in aqueous solution, is evidenced to bring about a "single-step four electron reduction" of 5-nitroimidazoles. As a result, species are expected to be sequentially generated within a small time scale. Hence, the damage caused to a target, that is, to nucleic acid bases or to calf thymus DNA, maintained in the immediate vicinity of the generation of reduced species may not be exclusively due to a particular species. While $RNO_2^{\bullet-}$ could have a substantial role, other reduction products formed during the electrochemical reduction of the compounds would also generate species that could modify targets. Because formation of $RNO_2^{\bullet-}$ is the first

Scheme 1. Probable Mechanism for the Interaction of the Nitro-Radical Anion with Thymine
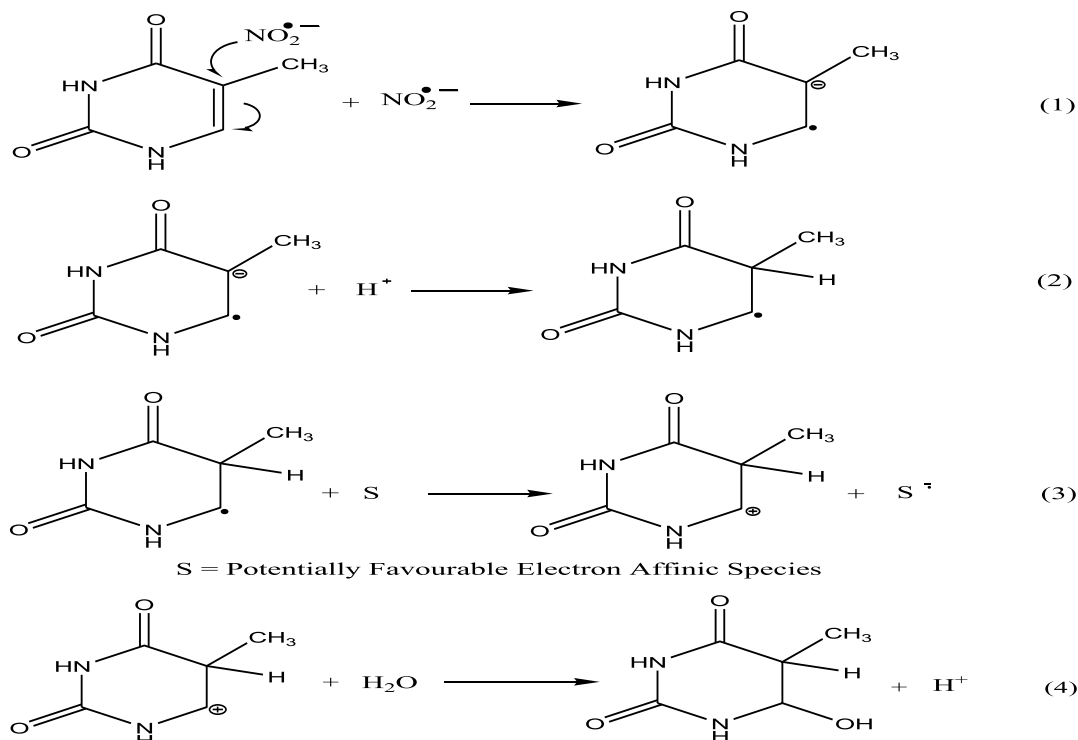

Table 1. Enhancement Ratio for the Damage Caused to Thymine Following the Reduction of tnz and Its Cu(II) Complexes at Respective Reduction Potentials in Aqueous Solution

sensitizer	loss of thymine from slope of degradation plot	enhancement ratio (for thymine)	loss of cytosine from slope of degradation plot	enhancement ratio (for cytosine)	loss of adenine from slope of degradation plot	enhancement ratio (for adenine)
	0.73		0.51		0.61	
tnz	0.86	1.18	0.54	1.06	0.67	1.10
Cu−tnz^{monomer}	1.43	1.96	0.65	1.27	2.11	3.46
Cu−tnz^{dimer}	2.30	3.15	1.64	3.22	1.69	2.77

step of the reduction process and being a radical, it is likely to have a high probability to interact with a target before being reduced to its next state. If the rate of interaction of $RNO_2^{•−}$ either with a nucleic acid base or with calf thymus DNA is higher than its tendency to be reduced further, interaction with $RNO_2^{•−}$ would be predominant. Hence, while other reduction products of a complex or of tnz could well be involved in a modification of the target, $RNO_2^{•−}$ might have a substantial contribution to the damage detected (Scheme 1, shown with respect to thymine).[27,35] This study was actually performed to realize how different reduction products generated electrochemically either on tnz or on its complexes with Cu(II) interact with nucleic acid bases and with DNA to realize what would happen when they are present within cells and undergo enzymatic reduction. For several decades now, the reduction of nitroimidazoles is considered very crucial for cytotoxic action for which they are much sought after.[1−3,5−14]

Again, considering the variety of species that are formed in solution, there is a good possibility for the formation of $RNO_2^{•−}$ either directly or through comproportionation, when an −NO₂ containing moiety (either on tnz or on a complex) interacts with another molecule that contains, say, −NHOH.[19,35,36,49,50] The possibility of disproportionation of $RNO_2^{•−}$, known to depend on pH, on the solvent and also on the material of the electrode also exist.[19,35,36,49,50] Hence,

depending on different reduction products, that in turn depends on whether they were generated on tnz present alone or on tnz present as part of a complex, substrates (nucleic acid bases or calf thymus DNA) interacting with $RNO_2^{•−}$ become a high possibility. If the rate of depletion of $RNO_2^{•−}$ in solution either due to disproportionation or in some other pathway is less, there is a good possibility of it interacting with a target maintained in its immediate vicinity. If, however, it is otherwise, then the interaction due to $RNO_2^{•−}$ would not be dominant, that is, it would not be the major cause of transformations either on nucleic acid bases or on calf thymus DNA. However, given the experimental design, although disproportionation is a possibility, it would only occur if the concentration of the species formed in solution are higher than that in our experiments. Under the conditions of the experiment, concentrations of electrochemically reduced species formed on tnz or its complexes would never be very high in solution; in fact, immediately after their generation, they would see more of the nucleic acid bases than one of its own kind (target/compound: 10:1); hence, the scope of disproportionation of $RNO_2^{•−}$ would be small.[19,35,36,49,50] Although explained qualitatively, $RNO_2^{•−}$ could eventually become an important species among other reduced products generated either on free tnz or on tnz present as part of a Cu^{II} complex that might interact with a target.

A comparison of the damage caused to nucleic acid bases (Table 1) or to calf thymus DNA (Table 2) reveals that the

Table 2. Enhancement Ratio for the Damage Caused to Calf Thymus DNA Following the Reduction of tnz and Its Cu(II) Complexes at Their Respective Reduction Potentials in Aqueous Solution

sensitizer	DNA double strand modification from slopes of degradation plots	enhancement^{ratio} (for DNA)
	0.0036	
tnz	0.0040	1.10
Cu−tnz^{monomer}	0.0053	1.45
Cu−tnz^{dimer}	0.0088	2.40

dimeric complex is the most effective. As can be seen from the structures of the two complexes (Figure 1), both have two units of tnz in them. Moreover, because it has been shown earlier that complex formation of tnz by Cu^{II} results in a decrease in nitro-radical anion formation;[20,21] hence, greater efficacy due to the dimeric complex and its difference with the monomeric one may not be due to the presence of tnz in the complexes. Rather, the dimeric complex having two Cu^{II} centers, against one in the monomer, could serve as a possible reason for the difference in activity. A greater presence of Cu^{II} in the dimer could be responsible for more interaction of the dimeric complex with thymine or cytosine or with calf thymus DNA via Cu^{II} that could help in the modification of the target or simply enable the compound to engage more with the target. Either way, a certain amount of thymine or cytosine or a certain amount of calf thymus DNA would not be detected by HPLC as free thymine or free cytosine (Table 1) or as free calf thymus DNA in a fluorescence-based EtBr experiment (Table 2). In the case of adenine, however, the monomeric complex performs much better which could be due to the larger size of the dimeric complex and that adenine, being a purine-based nuclei acid base, is also large.

Quite interestingly, trends observed in Tables 1 and 2 are similar, indicating that DNA having a greater percentage of thymine, similar to that in calf thymus DNA (41.9 mol % G-C and 58.1 mol % A-T), should be susceptible to a greater attack by the Cu^{II} complexes of tnz. Hence, a prior knowledge on the damaging ability of a compound on nucleic acid bases is extremely important because it helps one to use the correct compound in targeting a disease-causing microbe; at the same time, such prior knowledge also enables one to know the extent to which the compound could be harmful to the host, that is, whether it could affect the DNA of the host as well. Therefore, the findings of this study helps one to realize why tnz has been so successful against disease-causing microbes that have a high thymine content in their DNA (Table S1, Supporting Information).

In spite of decreased nitro-radical anion formation,[20,21] Cu^{II} complexes of tnz were found to be better in performance on nucleic acid bases and calf thymus DNA than tnz. This is also very interesting when for the 5-nitroimidazole family of drugs, nitro-radical anion formation is considered important for drug action. Therefore, it seems that the efficacy of the complexes are not due to free radical formation involving redox pathways that involve tnz; rather, a better performance by complexes is due to certain attributes of complex formation, those that involve the Cu^{II} center,[41,42] or due to interaction between the various constituents of DNA and the Cu^{II} center that is able to

cause double strand modification which is also detected by the technique used (i.e., the decrease in DNA-EtBr fluorescence).[38−42] Therefore, results of experiments with calf thymus DNA indicate they are probably not a consequence of the free radical activity involving tnz, rather other factors, such as the presence of Cu^{II} in the complex could well be involved.

To be sure about our model studies, an attempt was made to study the performance of the complexes, on their ability to prevent biofilm formation on *S. aureus* and *P. aeruginosa* that are responsible for causing nosocomial infections. Lower minimum bactericidal concentration (MBC) values for the monomeric and dimeric Cu^{II} complexes compared to tnz suggests better efficacy due to the complexes in removing biofilm cells. The in-dwelling bacterial cells within the biofilm matrix have a continuous and rich supply of nutrients and water molecules, much needed for their survival under stressed conditions due to the extremes of temperature, pH, salt concentration, or the presence of antimicrobials. The biofilm matrix also consists of lipid molecules, nucleic acids, proteins, extracellular DNA, and QS signaling molecules needed of cell density-dependent intercellular communications that are required for the growth of the biofilm and its sustenance. It was found that Cu^{II} complexes of tnz were able to bring about substantial changes in biofilm concentration both for *S. aureus* and *P. aeruginosa*; monomeric complex having a better efficacy against sessile colonies.

Biofilm-associated infections are found to occur via two mechanisms: (1) through biofilm formation by enhanced QS that occurs by the production of small signaling molecules capable of detecting the cell population density in the neighboring environment under stressed conditions and (2) by the spreading of microbial cells from the EPS matrix infecting newer places. From our study, we found that the monomeric complex has the ability to block the synthesis of signaling molecules responsible for regulating biofilm formation by inhibiting LasI/Rhl I synthase.[48] Thus, the monomeric complex has the potential of inhibiting the QS mechanism of *P. aeruginosa* by inhibiting QS-genes and blocking transcriptional regulatory proteins that inactivate LasR or RhlR systems.

While studying the interactions of tnz and its two complexes with nucleic acid bases and calf thymus DNA, it was revealed that the dimeric complex performs better, followed by the monomeric one and tnz. Therefore, it was expected that efficacy in prevention of biofilm formation would also follow the same trend. However, in case of biofilm-related experiments, we found that the monomeric complex was most efficacious to the pathogenic target, followed by the dimeric complex and tnz. Such an anomaly is not unexpected as nitro radical anions generated from tnz and its complexes vary widely. As observed in previous communications, complex formation is associated with quenching of nitro radical anion concentration.[20,21] We expect the monomeric complex to quench radical anion concentration in a manner just sufficient to eliminate the excess that would be responsible for toxic side effects, keeping the efficacious concentration of radical anions intact. This combined with improved binding with DNA over tnz is expected to give it the much superior boost for maximum efficacy. The dimeric complex, on the other hand, is expected to quench radical anion concentration more extensively due to the presence of two Cu^{II} centers; hence, more of the efficacious portion of the nitro radical anion concentration is eliminated. Moreover, owing to a larger size, efficacy of the dimeric

complex through binding is probably compromised; reason why in our model studies also the dimeric complex performs better on pyrimidine-based nucleic acid bases cytosine and thymine but not on purine-based adenine. The dimeric complex was however found to be more efficacious than tnz owing to attributes of complex formation.

The concentrations of compounds varied from one another in biological studies on biofilm formation and the model studies because in the case of prevention or eradication of bacterial biofilm formation and growth, emphasis was given to the obtained minimum inhibitory concentration (MIC) and MBC values, respectively. Hence, while antibacterial and antibiofilm studies were performed keeping in mind MIC and MBC values on sessile *P. aeruginosa* (which for the monomeric Cu^{II} complex was 20.25 μM and for the dimeric complex, 45.0 μM), for the model studies, slightly higher concentrations were used because for the model studies, where the technique employed was electrochemical reduction, if sufficient material is not present, the species generated might not be adequate for interaction with nucleic acid bases or with DNA.

The expression of biofilm-forming bacterial genes is regulated by a cell-population density-dependent mechanism known as QS. Both Gram-negative and Gram-positive bacteria perform QS by the mechanism of small signal molecules that varies from Gram-negative to Gram-positive bacteria. *N*-Acyl homoserine lactone (AHL) molecules (autoinducer-1, AI-1) are widely detected in Gram-negative bacteria, while for Gram-positive bacteria mainly peptides [autoinducer peptides (AIP) or QS peptides] are used.[49] We also checked the expression of virulence factors such as pyocyanin production, elastase, las A protease, and las B elastase in *P. aeruginosa* (Gram-negative) in the presence of the monomeric form of the compound. This indicates the modulation and prevention of the biofilm forming a signaling network in the presence of antimicrobial agents. However, the expression of virulence factors in Gram-positive bacteria such as *S. aureus* is directly linked to alterations in expression profiles of peptides/proteins such as endotoxins, haemolysins, exotoxins, autoinducing peptide 2 (AIP 2), proteases, and so forth that were not monitored as a part of this study.[50]

Our main aim was to highlight antibiofilm properties of copper(II) complexes by the formation of electrochemically generated nitro radical anion triggering bacteria-mediated enzymatic reduction. For this purpose, we only showed alterations in QS mechanism in *P. aeruginosa*. Alteration in biofilm formation and growth is also affected in the presence of copper(II) complexes in *S. aureus* as realized from Figure 8 that clearly depicts the reduction of EPS components.

CONCLUSIONS

Tnz is reported to bind to DNA while inside a cell initiating cytotoxic action on a pathogen by forming nitro radical anion, considered responsible for its efficacy. Excess production of such nitro radical anion is responsible for idiosyncratic side effects which metal complexes with reduced formation might control. Hence, both from model studies and from the prevention of biofilm formation, it may be said, what the complexes compromise in the free radical pathway, they make up through aspects such as better interaction with a target or due to the redox active Cu^{II}/Cu^{I} couple. Hence, Cu^{II} complexes of tnz, on the one hand, by controlling the generation of RNO$_2$$^{\bullet-}$, might control neurotoxic side effects, and on the other hand, continue to be better cytotoxic agents

than parent 5-nitroimidazoles (here, tnz) when one actually might expect them to have compromised on efficacy. This was clearly realized with the help of model studies using thymine, cytosine, adenine, and calf thymus DNA as targets as well as through studies on the prevention of biofilm formation. Such electrochemically generated species using compounds under consideration mimics what happens when the compounds are actually reduced within cells, helping one to understand the mechanism by which compounds impart biological efficacy.

EXPERIMENTAL SECTION

Materials and Methods. *Chemicals Used.* Tnz was purchased from Sigma-Aldrich and purified by re-crystallization from methanol. Copper(II) chloride (CuCl$_2$·2H$_2$O), copper(II) acetate [Cu(OAc)$_2$·H$_2$O], NaCl, NaNO$_3$, trichloroacetic acid (TCA), glacial acetic acid, sodium dihydrogen phosphate, disodium hydrogen phosphate, anthrone as reagent, Folin-Ciocalteu as reagent, congo red, cetyltrimethylammonium bromide (CTAB), chitin flakes, Tris—HCl, β-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), and KCl (all AR grade) were purchased from E. Merck, India. Thymine, cytosine, and adenine were purchased from TCI, Japan, and calf thymus DNA, crystal violet (CV), ethyl acetate, hydroxyl amine, NaOH, and ferric chloride were procured from Sisco Research Laboratories, India. Calf thymus DNA was dissolved in triple distilled water in the presence of 120 mM NaCl, 35 mM KCl, and 5 mM MgCl$_2$. Its concentration was determined using a molar extinction coefficient of 6600 M^{-1} cm^{-1} at 260 nm. Absorbance of the DNA solution was also measured at 280 nm; A_{260}/A_{280} was determined. The value found in the range 1.8−1.9 was considered ready for use, not requiring further purification. Quality of calf thymus DNA was verified using circular dichroism (CD), recording its response at 260 nm on a CD spectropolarimeter (J815—JASCO, Japan). Aqueous solutions of all other substances were prepared in triple distilled water.

Synthesis of [Cu^{II}(tnz)$_2$Cl$_2$] and [Cu$^{II}_2$(OAc)$_4$(tnz)$_2$]. A solution of tnz (0.494 g in 25 mL, 2.00 mmol) in methanol was gradually added with stirring to a solution of CuCl$_2$·2H$_2$O (0.17 g in 25 mL, 1.00 mmol) in methanol.[21] The mixture was warmed under reflux to ∼60 °C for 6 h. A green crystalline monomeric compound was obtained after 10 days following slow evaporation of the solvent.[21] A solution of tnz (0.494 g in 25 mL, 2.00 mmol) in methanol was gradually added with stirring to a solution of Cu(II) acetate (0.400 g in 25 mL, 2.00 mmol) in mildly warm methanol.[24] The mixture was warmed under reflux to ∼55 °C for 8 h. A dimeric Cu(II) complex of tnz was obtained after a week's time following slow evaporation of the solvent.[24] Both complexes were purified and crystallized.

Electrochemical Measurements. Electrochemical experiments were performed in an air-tight 50 mL electrochemical cell. Voltammograms were recorded on a Metrohm—Autolab PGSTAT 101 potentiostat. Analyses of data were done using the NOVA 1.10.1.9 program. A conventional three-electrode system, glassy carbon as the working electrode, platinum wire as the counter electrode, and Ag/AgCl, satd. KCl as the reference electrode were used. Solutions were degassed for ∼30 min prior to an electrochemical experiment using high-purity argon. Reduction of the nitro group in both monomeric and dimeric Cu^{II} complexes of tnz and on tnz itself were followed in aqueous, aqueous-dimethyl formamide (DMF), and pure DMF solvents using cyclic voltammetry. In the case

of pure DMF, the electrolyte was tetrabutyl ammonium bromide, while for aqueous solutions, it was KCl. In DMF, there is initially one-electron reduction to $NO_2^{\bullet-}$ that subsequently undergoes three-electron reduction to $-NHOH$.[35,36] As the percentage of water increases, the clarity of two reduction peaks is lost, and in purely aqueous solution, a single-step four electron reduction occurs. Results were also analyzed by the Randles−Sevcik equation because this confirms that the process is diffusion controlled (eq 2), an important prerequisite for experiments performed in this study.[35,51,52]

$$i_{pc} = (2.69 \times 10^5) \cdot n^{3/2} \cdot D_0^{1/2} \cdot A \cdot C \cdot \nu^{1/2} \tag{2}$$

i_{pc} refers to the current in amperes at the cathodic peak potential, n denotes the total number of electrons, D_0 is the diffusion coefficient of species, and A refers to the area of electrode in cm^2; surface area of the glassy carbon electrode used was 0.1256 cm^2. C refers to the concentration of compounds in moles/cm^3 and ν, the scan rate in V s^{-1}. Most of these parameters would have a role to play in the subsequent reduction of each compound performed in the presence of nucleic acid bases or calf thymus DNA.

Interaction of Reduced Products of tnz, [Cu(tnz)$_2$Cl$_2$], and [Cu$^{II}_2$(OAc)$_4$(tnz)$_2$] with the Target. The glassy carbon electrode maintained at a previously determined reduction potential of each compound helped to electrochemically generate different reduction products in aqueous solution that includes $RNO_2^{\bullet-}$ under de-aerated (Ar saturated) conditions. Because in the immediate vicinity of such in situ generated reduction products, thymine or cytosine or adenine or calf thymus DNA were maintained (separately), and they got an opportunity to interact with the species generated.[26,27,53] Time for in situ electrochemical generation of reduced species either on the monomeric or dimeric complexes or on tnz was strictly maintained constant for a certain target so that results obtained for nucleic acid bases and calf thymus DNA, due to each compound used could be compared with regard to species generated in solution.[54,55] The generated species bring about a change on the target maintained in the immediate vicinity of their generation.[53−55] Using the same experimental setup in aqueous solution, reduction of tnz was carried out at −0.745 V (pH 7.4), the monomeric complex at −0.700 V (pH 7.4), and the dimeric complex at −0.710 V (pH 7.4). The nucleic acid bases or calf thymus DNA were each investigated following interaction with reduced products formed either on tnz or on tnz present as a ligand in the complexes.[26,27,53] Concentrations of compounds used in the study were 1/10 that of the target (nucleic acid bases or calf thymus DNA). Control experiments were performed where aqueous solutions of nucleic acid bases or calf thymus DNA (without any compound) were subjected to a constant potential of −0.700 V at pH 7.4 using the same glassy carbon electrode.[26,27]

The amount of nucleic acid bases remaining was determined using HPLC. A C-18 column was used as the stationary phase and 5% aqueous-methanol as the mobile phase.[26,27] Amount of calf thymus DNA remaining unaltered was determined by treating it with ethidium bromide (EtBr) and subsequently determining the fluorescence of the adduct on a RF-530 IPC Spectrofluorophotometer, Shimadzu, Japan.[26,27] Interaction of EtBr with DNA leads to an increase in fluorescence, a fact that was utilized in this case to determine the amount of DNA

remaining intact following interaction with electrochemically generated reduced species.[38−42]

Determination of MIC and MBC. MBC values of monomeric and dimeric complexes of Cu^{II} against *P. aeruginosa* ATCC and *S. aureus* ATCC were determined by micro-dilution techniques.[56] Bacterial cells were inoculated in microtiter plates at a concentration of 10^6 CFU/mL in a volume of 50 mL. Complexes of varying concentrations were added separately and incubated at 37 °C for 24 h. Afterward, they were analyzed at 600 nm using a spectrophotometer. Antibacterial efficacy of monomeric and dimeric complexes was analyzed by determining the diameter of the zone of inhibition in millimeters. Sterilized discs of paper soaked in various concentrations of monomeric and dimeric complexes were placed on agar plates possessing *P. aeruginosa* and *S. aureus,* followed by the determination of clear zones of inhibition. Susceptibility of microbial strains to antimicrobial agents was determined by calculating the zone of inhibition as per recommendations of the National Committee for Clinical Laboratory Standards.[57]

Formation of *P. aeruginosa* and *S. aureus* Biofilm. Formation of biofilm by *P. aeruginosa* and *S. aureus* was determined using 96 polystyrene well plates for a period of 72 h at 37 °C, followed by washing with phosphate buffer and staining with 0.4% (v/v) CV, dissolved in glacial acetic acid 30% (v/v) for 10 min. It was then allowed to dry for 30−45 min, followed by rinsing with phosphate buffer. Subsequently, it was allowed to dry at room temperature for approximately an hour. A 33% (v/v) acetic acid solution was added and optical density (OD) was measured at 540 nm using a spectrophotometer.

Assay of Antibiofilm Activity. Rate of inhibition of biofilm formation achieved by the action of the monomeric and dimeric Cu^{II} complexes of tnz at MBC, incubated at 37 °C for 72 h was detected by the CV assay.[56,58]

Percentage inhibition was measured with respect to untreated control using the formula mentioned in eq 3.

$$
\begin{aligned}
&\text{Percentage biofilm inhibition} \\
&= \frac{[(\text{OD of untreated control}) - (\text{OD of treated sample})]}{(\text{OD of untreated control})} \\
&\times 100
\end{aligned}
\tag{3}
$$

Detection of QS in test *P. aeruginosa* and *S. aureus*

The supernatant of bacterial culture broth was filtered using a membrane filter having pore size 0.2 μm. Ethyl acetate was added to the filtrate with gentle shaking for 10 min to allow for phase separation.[56,58] The upper fraction of the mixture was mixed with 2 M hydroxyl amine and 3.5 M NaOH (1:1), followed by 10 μL of alcoholic ferric chloride solution (ferric chloride in 95% 1:1 ethanol). Color of the solution was measured with a spectrophotometer at 520 nm.[59]

Quantification of Secondary Metabolite Pyocyanin Produced by *P. aeruginosa* during Biofilm Formation. Quantification of pyocyanin[60] produced by *P. aeruginosa* upon incubation with MBC concentrations of monomeric and dimeric Cu^{II} complexes and amoxicillin (standard antibiotic) was done at 37 °C for 48 h. The culture supernatant (5 mL) collected after centrifugation at 10,000 rpm for a period of 15 min[61] was added to 3 mL of chloroform, followed by re-extraction with 1 mL of 0.2 N HCl, resulting in a color change from orange to pink that was detected at 520 nm using a

spectrophotometer. This helped in determining the percentage reduction of pyocyanin.

Determination of Elastase Activity. Quantification of *las B* expression was done by determining the elastase activity. An aliquot of culture supernatant (100 μL) was added to 900 μL of Elastin Congo red (ECR) and incubated at 37 °C for 3 h. Insoluble ECR was removed by centrifugation and absorbance was measured at 495 nm.[62]

Determination of Rhamnolipid Production and Drop Collapse Assay. The amount of rhamnolipid was estimated with CTAB-methylene blue plates in accordance with a method described earlier.[63,64] Plates were supplemented with 0.2% (w/v) CTAB, 0.0005% (w/v) methylene blue, and solidified with 1.5% (w/v) agar. An overnight grown liquid culture of *P. aeruginosa* was used and a spot was applied at the middle of the plate for swarming assays. To all plates, except control, monomeric, and dimeric, Cu^{II} complexes were added separately. Plates were incubated at 37 °C for 24 h, followed by incubation at room temperature for another 24 h. Production of rhamnolipid was estimated by measuring the dark blue halo surrounding the colony and quantification was done following a protocol described earlier.[65]

Detection of Viability Count of the Sessile Group of Bacterial Cells. The working strain grown on 0.1% chitin flakes (w/v) for 72 h was washed with 0.1% (w/v) normal saline to eliminate planktonic groups of cells. Following the treatment of sessile cells as control or with monomeric and dimeric Cu^{II} complexes, bacterial growth was determined at 590 nm using a spectrophotometer at varying intervals of time.[66]

Determination of EPS Degradation on Being Challenged by Monomeric and Dimeric Cu^{II} Complexes. Biofilms of the working strain were grown on chitin flakes 0.1% (w/v) separately in 100 mL of LB media and centrifuged at 12,000 rpm for 15 min at 4 °C to break the biofilm. 5 mL of PBS buffer was used to wash the pellets collected after centrifugation and mixed with 2.5 mL of 10 mM Tris−HCl (pH 7.8). After thorough cyclomixing, 20 mM β-mercaptoethanol and 1 mM PMSF were added. The cell suspension of bacterial culture was sonicated, followed by centrifugation (12,000 rpm, 30 min) at 4 °C, followed by the addition of 10% TCA in acetone.[67]

Estimation of Carbohydrate and Protein Content in EPS when Challenged by Monomeric and Dimeric Complexes of Cu^{II}. The carbohydrate present in EPS was quantified using the Anthrone method.[68] Protein present in EPS was quantified by the Lowry method.[69]

Isolation and Estimation of DNA from Prokaryotic Cells. To have a check on adverse effects related to the use of monomeric and dimeric Cu^{II} complexes on genomic DNA of bacterial strains, they were isolated using CTAB after treatment with monomeric and dimeric complexes for 2 h keeping the "control" untreated. Concentration of DNA was measured spectrophotometrically at 260 nm and quantified as in eq 4.

$$\text{Units}(\ \times\ )\text{mg mL}^{-1}$$
$$= 50 \times \text{OD at 260 nm} \times \text{dilution factor} \qquad (4)$$

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c04822.

> HPLC chromatograms for 10^{-3} M thymine, cytosine, and adenine, respectively, recorded at 254 nm to denote the region of elution of the three nucleic acid bases and activity of the drug Tindamax used to treat different types of disease-causing organisms along with the composition of A-T and G-C content of their DNA (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Saurabh Das − *Department of Chemistry, Inorganic Chemistry Section, Jadavpur University, Kolkata 700 032, India;* ⓞ orcid.org/0000-0002-0455-8760; Phone: +91 33 24572148; Email: saurabh.das@jadavpuruniversity.in, dasrsv@yahoo.in; Fax: +91 33 24146223

Authors

Promita Nandy − *Department of Chemistry, Inorganic Chemistry Section, Jadavpur University, Kolkata 700 032, India*

Ramesh C. Santra − *Department of Chemistry, Inorganic Chemistry Section, Jadavpur University, Kolkata 700 032, India*

Dibyajit Lahiri − *Department of Biotechnology, University of Engineering and Management, Kolkata 700 156, India*

Moupriya Nag − *Department of Biotechnology, University of Engineering and Management, Kolkata 700 156, India*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acsomega.1c04822

Author Contributions

The manuscript was written through contributions made by all authors. All authors have given their approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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