# A Copper-Metalated, Hybrid Inorganic–Organic Polymer as an Oxidative Nuclease

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*Dedicated to Professor S. S. Krishnamurthy on the occasion of his 63rd birthday*

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This study describes nucleolytic activity of a copper-metalated, multisite-coordinating, hybrid polymer **CPPL-Cu** under oxidative conditions. Rapid relaxation of supercoiled plasmid DNA was observed and mechanistic probing by chemical and enzymatic assays revealed involvement of singlet-oxygen-derived reactive species. The heterogeneous nature of the catalyst allowed a facile recycling of the artificial nuclease.


## Introduction

As many phosphate-ester-modifying enzymes depend on metal ions for their catalytic activities, it is not surprising to find that numerous artificial phosphatases and nucleases-containing metal ions have been reported over the past few decades for possible biochemical applications ranging from chemotherapy to synthetic restriction enzymes.[1] These model systems employ a wide range of transition and inner-transition-metal ions coordinated to ligands of natural and synthetic origin. Although enzyme-like substrate recognition, efficiency, and high turnover remain elusive with such systems, they provide incisive insight to the study of the role of metal ions for phosphate ester modification in natural enzymes.[2–5]

Among the many transition-metal ions, copper-based artificial systems have received considerable focus perhaps due to their variable mechanism of reactivity: they can modify natural and non-natural phosphate esters either through an oxidative route or a hydrolytic pathway. The former activity can be attributed to the redox cycling of copper in the presence of exogenously added oxidants or reductants, which leads to in situ formation of reactive species.[6]

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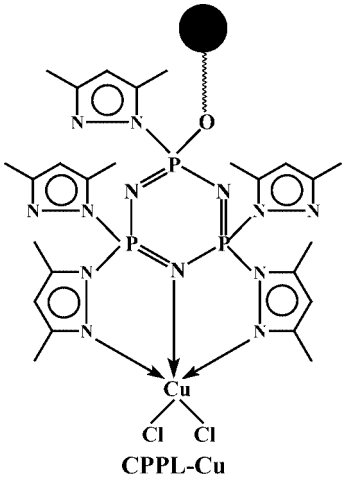
Polyphosphazenes, $[N{=}PR_2]_n$, are the most versatile and well-studied family of inorganic polymers.[7] One of the remarkable features of this class of polymers is that individual polymer properties can be readily modulated by a variation of the substituent on the phosphorus center.[8–10] Several applications of these polymers in diverse fields[11–14] are known, including the use of these polymers as matrices for stabilizing gold nanoparticles.[15] A closely related class of hybrid inorganic–organic polymers contains an intact cyclophosphazene ring as a pendant group attached at regular intervals to the backbone of an organic polymer.[16–18] Although much less studied, these polymers also show considerable promise particularly in the design of polymeric ligands.[19–20] We have recently utilized this approach to design new polymeric catalysts, which show applications in phosphate ester hydrolysis[21] or in the Heck arylation reaction.[22] In the following account we report the first application of these hybrid inorganic–organic polymers in DNA cleavage under heterogeneous reaction conditions.

## Results and Discussion

A number of inner and outer-transition-metal ions are known for their catalytic assistance towards nucleic acid cleavage, either by a hydrolytic pathway[23–31] or through oxidative damage.[32–35] In particular, copper complexes afford facile nucleic acid oxidative cleavage in the presence of oxidizing and reducing agents,[36–43] in addition to the hydrolytic mechanism.[23,27,29] Most of these artificial phosphatases and nucleases offer homogeneous catalysis, while

only limited applications of heterogeneous catalysts have been reported.[44–52]

We have utilized copper-metalated, pyrazolylcyclotriphosphazene-containing, cross-linked polymeric catalyst CPPL-Cu (Scheme 1) for the oxidative damage to supercoiled plasmid in the presence of oxidizing agents such as magnesium monoperoxyphthalate (MMPP) and oxone. To the best of our knowledge, this is the first instance where an inorganic–organic hybrid polymer, and more specifically, a polyphosphazene-appended polymeric system has been used as a synthetic nuclease. The molecular structure of CPPL-Cu, has been previously established by us.[21] Briefly, the EPR spectrum of CPPL-Cu at 77 K is of the axial type and the parameters obtained viz., $g_{II} = 2.27$, $g_{\perp} = 2.10$, and $A_{II} = 130.5 \times 10^{-4}\ cm^{-1}$, compare very well with that of model compounds $N_3P_3(3,5\text{-}Me_2Pz)_5(O\text{–}C_6H_4\text{–}C_6H_4\text{–}CH{=}CH_2)\cdot CuCl_2$ [$g_{II} = 2.28$, $g_{\perp} = 2.05$, and $A_{II} = 130.5 \times 10^{-4}\ cm^{-1}$] and $N_3P_3(3,5\text{-}Me_2Pz)_5(O\text{–}C_6H_4\text{–}CHO)\cdot CuCl_2$ [$g_{II} = 2.28$, $g_{\perp} = 2.06$, and $A_{II} = 122.0 \times 10^{-4}\ cm^{-1}$].




**CPPL-Cu**

Scheme 1.

The X-ray crystal structure of the model compound $N_3P_3(3,5\text{-}Me_2Pz)_5(O\text{–}C_6H_4\text{–}CHO)\cdot CuCl_2$ has been determined and shows a five-coordinate environment around $Cu^{II}$ containing a cyclophosphazene ring nitrogen atom, two pyrazolyl nitrogen atoms and two chlorine atoms. Further, the diffuse reflectance spectrum of CPPL-Cu shows peaks at 845, 351, 318, 304, and 251 nm. These peak positions are similar to those observed in the solution spectrum of the model compounds. On the basis of this cumulative data, it is suggested that the coordination environment around copper contains two chlorides along with two nongeminal pyrazolyl nitrogens and one cyclophosphazene-ring nitrogen. From the nitrogen analysis it is concluded that every gram of the polymer contains 0.33 g of the cyclophosphazene monomer corresponding to $4.09 \times 10^{-4}$ moles. From the AAS analysis the amount of copper present per gram of CPPL-Cu was found to be 21.7 mg.[21]

Plasmid (pBR322) modification assisted by the CPPL-Cu complex was studied in the presence of exogenously added co-oxidants MMPP and oxone. Initial experiments were performed using a varying amount of polymer in the presence of MMPP to study the concentration-dependent cleavage of pBR322. These experiments revealed a dependence of polymer concentration on the DNA cleavage (Figure 1). The concentration of catalyst for all subsequent experiments was optimized at 50 μg/20 μL for a 20-min cleavage reaction to set complete relaxation of plasmid DNA.

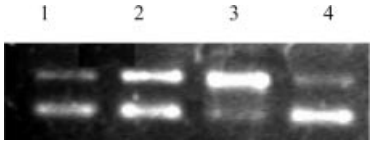



Figure 1. pBR322 cleavage with CPPL-Cu in presence of different amounts of copper polymer [lane 1: DNA + 10 μg of catalyst + MMPP (25 min); lane 2: DNA + 20 μg of polymer + MMPP (25 min); lane 3: DNA + 30 μg of polymer + MMPP (25 min); lane 4: DNA alone].

Both of the peracids effectively activated CPPL-Cu and a complete conversion of supercoiled plasmid DNA, from form I to nicked form II, and linear form III was observed (Figure 2, lanes 6 and 8). Control experiments with unmetalated ligand complex and peracid alone did not produce any detectable strand scission (Figure 2, lanes 2, 3, 5, and 7 respectively). However, the addition of EDTA afforded complete inhibition of plasmid cleavage, thus indicating the crucial role of coordinated copper ions in the cleavage reaction (Figure 2, lane 4).

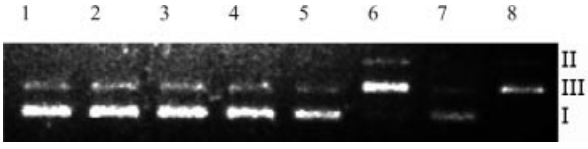



Figure 2. pBR322 cleavage by CPPL-Cu in the presence of MMPP and oxone (lane 1: DNA alone; lane 2: DNA + CPPL; lane 3: DNA + CPPL-Cu; lane 4: DNA + CPPL-Cu + EDTA + MMPP; lane 5; DNA + MMPP; lane 6: DNA + CPPL-Cu + MMPP; lane 7: DNA + oxone; lane 8: DNA + CPPL-Cu + oxone).

The nature of the reactive species responsible for DNA cleavage was probed by performing the reactions in the presence of hydroxyl-radical scavengers such as *tert*-butyl alcohol, dimethyl sulfoxide, and D-mannitol. These reagents partially inhibited the cleavage reaction (Figure 3; lanes 3–5), thus suggesting the possible involvement of reactive radical species in DNA cleavage. The extent of inhibition is limited probably because of the insoluble nature of the catalyst, which may shield the reactive species from freely diffusing out in the solution. Such protection has recently been observed with a trinuclear copper(II) complex where the presence of standard free-radical scavengers failed to reveal any interference against oxidative DNA cleavage.[53] As discussed in the literature, abstraction of C-4′ hydrogen appears to be the most likely mechanism of action for the reactive species so generated, leading to backbone scission.[54]

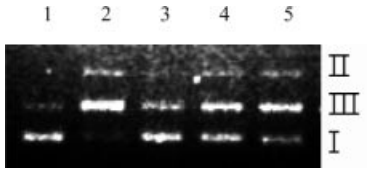



Figure 3. pBR322 cleavage by CPPL-Cu in the presence of radical scavengers (lane 1: DNA alone; lane 2: without the radical scavenger; lane 3: with *t*BuOH; lane 4: with DMSO; lane 5: with D-mannitol).

Enzymatic scavengers such as superoxide dismutase and catalase failed to provide any inhibition of the reaction, thereby suggesting a lack of involvement of the superoxide radical anion and the peroxide species in the cleavage (Figure 4, lanes 3, 4). Curiously, sodium azide afforded almost complete inhibition of plasmid relaxation, and the deployment of anaerobic conditions resulted in partial plasmid modification (Figure 4, lanes 5, 6). Taken together, these observations point towards a possible role of singlet oxygen in DNA modification as sodium azide is an effective quencher of singlet oxygen and some other reactive oxygen species. These results are similar to those of the hydroquinone/$Cu^{II}$ system where the DNA strand cleavage was partially inhibited by HO·scavengers, whereas a greater degree of protection was offered by singlet-oxygen scavengers.[55] This clearly suggests localized generation and attack of singlet oxygen or a related species, because of the possible formation of a copper-peroxide complex, rather than the predominance of HO·in DNA cleavage. A dual mechanism involving hydroxyl radicals and singlet oxygen for DNA cleavage using photoactivated copper-based catalysts has been recently reported by Chakravarty and coworkers.[56]

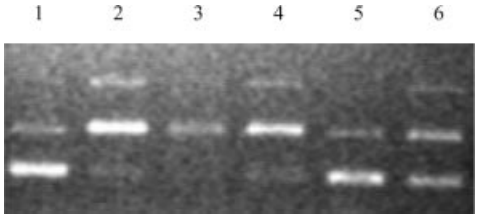



Figure 4. Lane 1: DNA alone; lane 2: DNA + CPPL-Cu + MMPP; lanes 3–5: DNA + CPPL-Cu + MMPP + SOD, catalase, sodium azide, respectively; lane 6: DNA + CPPL-Cu + MMPP under anaerobic conditions.

Growing interest in the development of heterogeneous nucleolytic reagents[44–46,48,51] and their possible application as recyclable catalytic systems has prompted us to explore CPPL-Cu for multiple cleavage reactions. Typically, reaction mixtures were centrifuged after completion of a reaction to leave the catalyst in the form of a pellet. After the removal of the supernatant, the pellet was repeatedly washed with cacodylate buffer ($4 \times 100$ μL) to ensure complete removal of DNA and then reused for subsequent plasmid modification. Gel electrophoretic tracking was used to detect the complete removal of plasmid and cleaved fragments. CPPL-Cu was recycled for three consecutive reactions and each time a complete conversion of supercoiled form I to linear form III was observed (Figure 5) thus making an emphatic statement about the robust nature of the CPPL-Cu catalytic system.

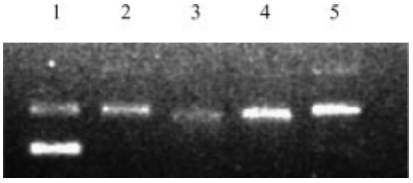



Figure 5. Recycling experiment [lane 1: DNA alone; lane 2: DNA + CPPL-Cu + MMPP (fresh); lane 3: first recycle; lane 4: second recycle; lane 5: third recycle].

We therefore perceive that the role of the cross-linked polymeric matrix is twofold: firstly, stabilization of the coordination complex by providing a suitable hydrophobic environment and secondly, providing an accessible, insoluble embedded metal complex scaffold for participation in the catalytic reactions. The latter property manifests itself in the facile recycling of the catalyst. As this catalyst relaxes plasmid superhelicity, it can also be considered to act as an artificial DNA topoisomerse. Such enzymes catalyze DNA strand scission releasing superhelical stress and religating activities in order to modulate nucleic acid architectures.[57] However, unlike topoisomerases our catalyst has not been optimized for the religation activity and thus, a direct comparison of their respective cleavage/ligation activities will not be meaningful.

## Conclusions

In summary, we have demonstrated a novel, effective, and hitherto unreported nucleolytic activity of hybrid polymers with biomolecules, the cleavage of supercoiled plasmid with **CPPL-Cu** by an oxidative mechanism. It was further verified that **CPPL-Cu** is an extremely robust and stable catalyst, and it can be recycled several times over because of the heterogeneous nature of the catalysis. This represents the first application of the cyclophosphazene pendant polymeric system in a biological milieu. It is possible to harness this motif efficiently for the coordination of diverse metal ions to create a set of heterogeneously active catalytic reagents for chemico-biological applications.

## Experimental Section

**General Remarks:** Solvents and other general reagents used in this work were purified according to the standard literature procedures.[58] CPPL and CPPL-Cu were prepared according to the reported procedures.[21] Elemental analyses were carried out with a Carlo–Erba CHNSO 1108 elemental analyzer. The amount of copper present in the polymeric catalyst was determined by atomic absorption spectrometry (AAS) with an Integra XL AAS spectrometer. EPR spectra were recorded with a Varian 109E Line Century Series, X-band spectrometer, at liquid nitrogen temperature. IR spectra of KBr pellets were recorded with a Bruker Vector 22 FTIR spectrophotometer operating from 400 to 4000 $cm^{-1}$. Ab-

sorption spectra were recorded with a Shimadzu UV-160 spectrophotometer.

**Preparation of CPPL:** To a solution of $N_3P_3(3,5\text{-}Me_2Pz)_5(OC_6H_4\text{-}p\text{-}C_6H_4\text{-}p\text{-}CH{=}CH_2)$ (1.0 g, 1.2 mmol) in 1,2-dichloroethane (15 mL) was added 1,4-divinylbenzene (80%, mixture of isomers, 0.4 g, 2.5 mmol) and AIBN (0.03 g, 0.2 mmol). The solution was purged with argon for 45 min and was subsequently heated at 80 °C for 36 h. The cross-linked polymer obtained was filtered and washed with toluene (3 × 20 mL), dichloromethane (3 × 20 mL), methanol (3 × 20 mL), and acetone (3 × 20 mL), and dried thoroughly under vacuum at 40 °C. Yield: 1.04 g. IR (KBr) $cm^{-1}$: 3397 (s), 2923 (s), 1604 (s), 1573 (s), 1493 (s), 1443 (s), 1412 (s), 1372 (m), 1301 (s), 1218 (vs), 1089 (m), 1023 (m), 967 (m), 902 (m), 824 (m), 796 (m), 711 (w), 597 (m), 525 (m). Found: C 64.98, H 6.2, N 9.32. From the nitrogen analysis it was concluded that every gram of the polymer contained 0.54 g of the cyclophosphazene monomer which corresponded to $6.7 \times 10^{-4}$ moles.

**Preparation of CPPL-Cu:** To a solution of anhydrous cupric chloride (0.09 g, 0.7 mmol) in ethanol CPPL (1.0 g, 0.67 mmol of the monomer) was added and stirred for 24 h. The resulting metalated polymer was washed thoroughly with methanol (5 × 20 mL) to remove any unreacted cupric chloride and dried under vacuum at 40 °C. Yield: 1.2 g. IR (KBr) $cm^{-1}$: 3420 (m), 3020 (m), 2923 (s), 2853 (m), 1649 (w), 1603 (w), 1570 (w), 1492 (m), 1454 (m), 1418 (m), 1374 (w), 1240 (vs), 1187 (vs), 1047 (m), 964 (w), 903 (m), 824 (m), 795 (m), 709 (w), 588 (w), 540 (w). Found: C 55.40, H 5.21, N 6.39. From the nitrogen analysis it was concluded that every gram of the polymer contained 0.33 g of the cyclophosphazene monomer which corresponded to $4.09 \times 10^{-4}$ moles. From the AAS analysis the amount of copper present per gram of CPPL-Cu was found to be 21.7 mg. EPR (solid, 77 K): $g_{II} = 2.27$; $g_{\perp} = 2.10$; $A_{II} = 130.5 \times 10^{-4}\,cm^{-1}$. Diffuse reflectance UV/Vis [$\lambda_{max}$/nm (absorbance)]: $\lambda_{max}$ = 845 (0.355), 351 (0.294), 318 (0.891), 304 (0.878), 251 (0.920).

**pBR322 Cleavage Assay:** Plasmid cleavage reactions (20 min) were performed in sodium cacodylate buffer (10 mM, pH 7.5, 20 μL, 30 °C) containing supercoiled plasmid DNA pBR322 (9 ng/μL, New England Biolabs), CPPL-Cu (the concentration of copper was 0.85 mM, if the catalyst was completely soluble in buffer), and an oxidizing agent [magnesium monoperoxyphthalate (100 μM) or oxone (100 μM)]. Individual reactions were quenched by adding the gel loading buffer (5 μL) containing EDTA (100 mM), loaded onto agarose gel (0.7%), containing ethidium bromide (1 μg/mL), and were electrophoresed for 1.5 h (60 mA). Gels were imaged with the Bio-Rad Gel Documentation System 2000. Recycle experiments were performed in a similar fashion except that they were performed for 25 min to ensure complete conversion of the supercoiled form to the relaxed form.

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