Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Bioorganic & Medicinal Chemistry Letters 14 (2004) 1559–1562
Bioorganic & Medicinal Chemistry Letters

Metalated hybrid polymers as catalytic reagents for phosphate ester hydrolysis and plasmid modification

Vadapalli Chandrasekhar,* Pravas Deria, Venkatasubbaiah Krishnan, Arunachalampillai Athimoolam, Sanjay Singh, C. Madhavaiah, S. G. Srivatsan and Sandeep Verma*

Department of Chemistry, Indian Institute of Technology, Kanpur-208016, India

Received 21 October 2003; revised 22 December 2003; accepted 22 December 2003

Abstract—Pendant pyrazolylcyclophosphazene containing hybrid cross-linked polymer (**CPPL**) has been utilized for binding Zn(II). The metalated polymer (**CPPL–Zn**) has been found to be very effective catalyst for the hydrolysis of a RNA model phosphodiester substrate [2-(hydroxypropyl)-*p*-nitrophenyl phosphate, *h*NPP]. In addition, **CPPL–Zn** also cleaved supercoiled plasmid DNA pBR322 thus providing a novel structural motif of inorganic–organic hybrid polymers as synthetic nucleases.

Chlorocyclophosphazenes are excellent precursors for the construction of multi-site coordination ligands due to their robust framework and reactive periphery.[1] Thus, replacement of the P–Cl bond in $N_3P_3Cl_6$ by appropriate substituents containing donor atoms can afford a library of diverse ligand systems.[2] We have carried out extensive work on the pyrazolyl cyclophosphazenes $N_3P_3(3,5\text{-}Me_2Pz)_6$ and *gem*-$N_3P_3Ph_2(3,5\text{-}Me_2Pz)_4$.[3] More recently we have incorporated the pyrazolylcyclotriphosphazene structural motif as a pendant group in a cross-linked polymer, **CPPL**, and have shown its utility for binding Cu(II).[4] In this paper, we report the preparation, characterization and catalytic activity of **CPPL–Zn**.[5] The effectiveness of **CPPL–Zn** as a heterogeneous catalyst has been tested by choosing hydrolysis of a RNA model substrate [2-(hydroxypropyl)-*p*-nitrophenyl phosphate, *h*NPP]. We have also found that **CPPL–Zn** is effective towards cleaving supercoiled plasmid DNA pBR322 in the absence of other exogenous reagents. Though copper(II) has slightly higher Z_{eff}/r value[6] to polarize the coordinated water in lower pH, we have chosen zinc in view of its coordination flexibility. This study represents one of the foremost examples of a catalytic application for this widely studied family of pyrazolylcyclophosphazene polymer systems.

The synthetic strategy followed for the preparation of the multi-site coordinating cross-linked polymer **CPPL** and its zinc complex, **CPPL–Zn,** is outlined in Scheme 1. The amount of zinc incorporated in the polymeric matrix was estimated by atomic absorption spectroscopy and was found to be 11.5 mg/g of the polymer. The metal ion does not leach out of **CPPL–Zn** even after prolonged incubation in the reaction buffer used for the hydrolysis of the phosphate diester, suggesting high stability of the metal–ligand system. This is in contrast to the model compound $N_3P_3(3,5\text{-}Me_2Pz)_6{\cdot}ZnCl_2$ which decomposes in aqueous methanol. The proposed coordination environment around zinc(II) in **CPPL–Zn** is based on an X-ray structure reported in the literature.[7]

Hydrolysis of the RNA model substrate, *h*NPP assisted by **CPPL–Zn** was conveniently monitored by the time-dependent release of *p*-nitrophenolate anion ($\varepsilon_{400} = 1.65\times10^4\ M^{-1}cm^{-1}$)[8] and was found to proceed with a significant rate enhancement. Pseudo-first-order rate constant (k_{obs}) for the hydrolysis of *h*NPP ($1.52\times10^{-3}\ min^{-1}$) shows a 1000-fold rate enhancement over the uncatalyzed hydrolysis.[9] Importantly, the use of unmetalated cross-linked polymer **CPPL** as a control for the acceleration of phosphate ester hydrolysis under conditions similar to those used for **CPPL–Zn**, revealed a lack of rate enhancement, indicating a crucial role for

Keywords: Cyclophosphazene; Pendant polymer; Catalyst; Zinc; Phosphate ester; Hydrolysis.
* Corresponding authors. Tel.: +91-5122-597259; fax: +91-5122-597259; e-mail: vc@iitk.ac.in

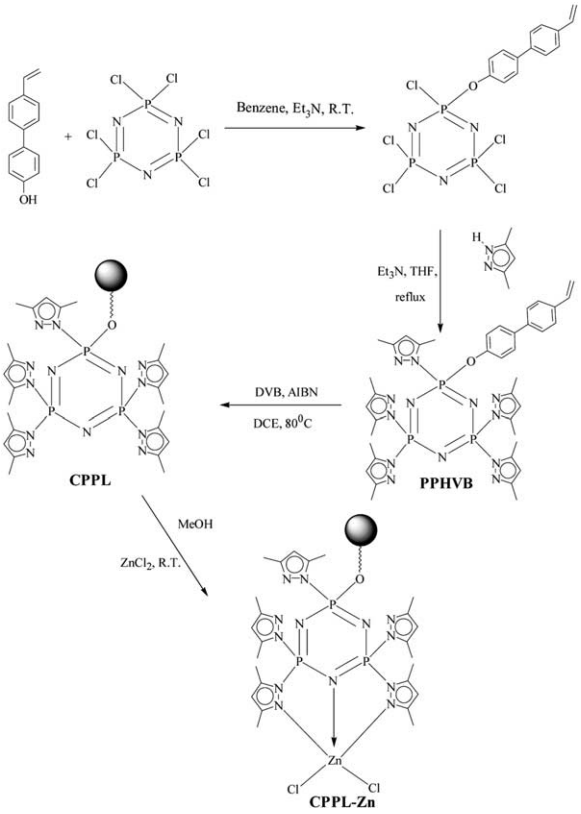
0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2003.12.082

coordinated zinc(II) ions in **CPPL–Zn**. This system followed typical saturation kinetics as evident from the V_i versus concentration of substrate plot (Fig. 1), thus prompting us to perform a thorough kinetic analysis. Michaelis–Menten kinetic parameters, K_m and V_{max}, were determined from the Lineweaver–Burk plot ($1/V_i$ versus 1/[S] plot, Fig. 2) and were found to be 1.53 mM and 3.2×10^{-3} mM min^{-1}, respectively.

A profile of the initial velocity (V_i) versus pH displayed a strong dependence of rate on pH variation. The beneficial role of transition metal ions in assisting phosphate ester hydrolysis is well documented.[10] We believe that the coordinated metal ion in our system activates the water molecule and electrostatically mitigates the negative charge on the phosphate moiety by its Lewis acidity.[11]

The mode of the phosphate ester hydrolysis assisted by our polymeric matrix was also studied by the time-dependant ^{31}P NMR analysis. Exclusive formation of cyclic phosphate with increasing peak intensity at 18.6 ppm, as a function of time, clearly indicates activation of internal hydroxyl group perhaps by the metal-bound hydroxide acting as a general base (Fig. 3).[12]

In order to demonstrate that the hydrolytic activity is solely due to zinc contained in the cross-linked polymer and not due to the leached out metal ions, the following experiments were carried out. In the reaction arrest experiment, **CPPL–Zn** was filtered from the reaction mixture after a reaction time of 4 h and the hydrolysis of *h*NPP was further monitored for 45 h. The hydrolysis was completely arrested as soon as the polymer was filtered, which strongly suggests that the hydrolytic activity is solely due to the coordinated zinc ions in the polymeric matrix (Fig. 4). Secondly, AAS analysis of the fresh and used catalyst confirmed that the metal ion has not leached out during the course of the catalytic



Scheme 1.

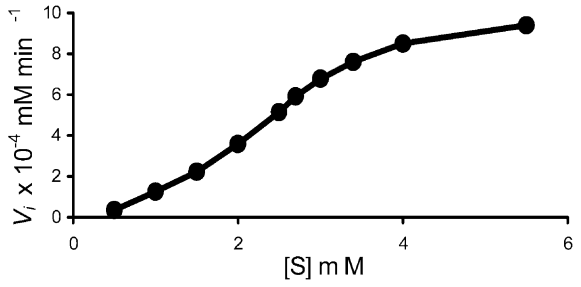


Figure 1. Saturation kinetics plot for *h*NPP hydrolysis.

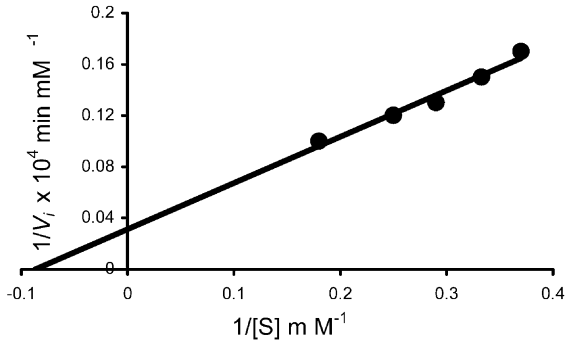


Figure 2. Lineweaver–Burk plot for *h*NPP hydrolysis.

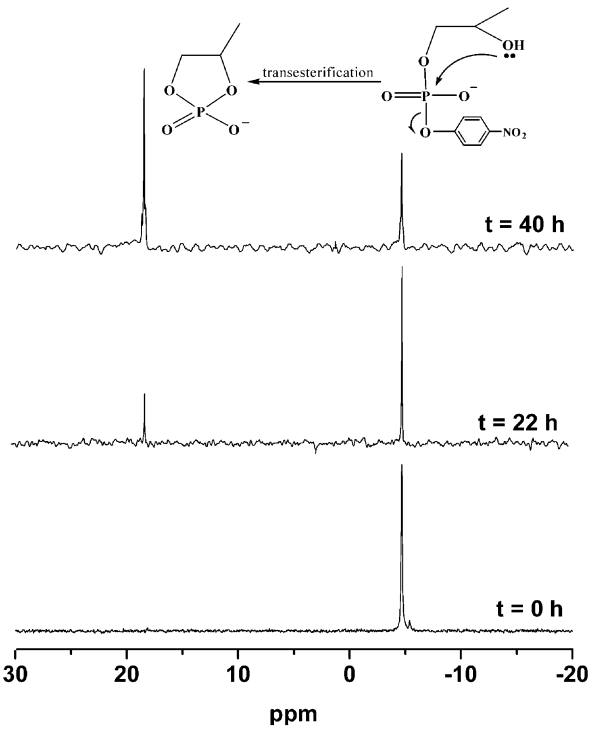


Figure 3. ^{31}P NMR spectra for the hydrolysis of *h*NPP by **CPPL–Zn**. Chemical shifts for *h*NPP and the cyclic phosphate product are −4.32 ppm and 18.6 ppm, respectively (ref 12d). The chemical shifts are with respect to 85% H_3PO_4 used as external reference.

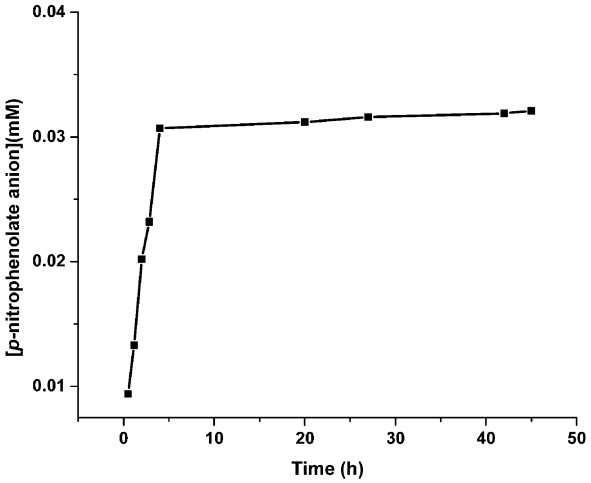


Figure 4. Reaction arrest experiment for *h*NPP hydrolysis.

reaction as in both cases the zinc content remained invariant. Thirdly, **CPPL–Zn** was separated from the reaction mixture, washed, dried and reused for the hydrolysis of *h*NPP under the same conditions. The initial velocities obtained for the catalyzed reaction with the reused catalyst are comparable with those obtained for the fresh catalyst (Table 1) indicating that **CPPL–Zn** retains its catalytic behavior through several cycles of hydrolytic reaction.

Attempts were made to explore the nuclease activity of **CPPL–Zn** towards the cleavage of supercoiled plasmid pBR322. The cleavage reaction was performed by suspending the polymer in cacodylate buffer containing pBR322.[13] In a 24 h reaction, there was nearly ~30% conversion of supercoiled DNA form I to nicked form II (Fig. 5). Here, it is important to mention that the polymeric system worked under heterogeneous reaction conditions and it did not require any external activating agent to produce observed DNA cleavage in contrast to numerous transition metal ion based complexes, which require exogenous oxidants to induce strand scission.[14] However, **CPPL–Zn** did not produce any detectable cleavage of lysozyme even when incubated for prolonged time periods (Fig. 6).[15] Heterogeneous nature of DNA cleavage is a useful observation and this property can be harnessed in devising possible applications in selective degradation of nucleic acid contaminants in various biological applications.

Table 1. Recycle experiment for *h*NPP hydrolysis with **CPPL–Zn**[a]

Catalyst	$V_i \times 10^{-4}$ (mM min^{-1})
Fresh **CPPL–Zn**	0.67
First recycle	0.52
Second recycle	0.58
Third recycle	0.67

[a] All hydrolytic reactions were performed in duplicate in 0.01 M *N*-ethyl morpholine buffer (pH 8.0, T = 30 °C), prepared in 50% aqueous methanol. Weight of the polymer used was 3 mg/3 mL, corresponding to 0.17 mM of Zn if the polymer were to be completely soluble in the buffer. Concentration of the *h*NPP was 0.5 mM.

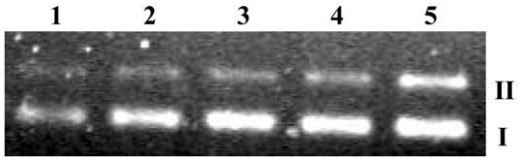


Figure 5. Cleavage of supercoiled plasmid DNA pBR322 by **CPPL–Zn**. Lane 1: DNA alone; Lane 2: DNA alone (24 h); Lanes 3-5: DNA + **CPPL–Zn** (12, 16, 24 h respectively).

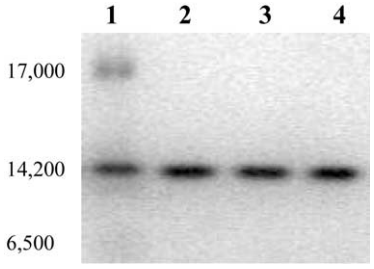


Figure 6. Lysozyme cleavage experiment by using **CPPL–Zn**. Lane 1: Molecular weight markers (Da) (17,000; 14,200; 6,500 from top to bottom); Lane 2: Lysozyme alone; Lanes 3, 4: Lysozyme with polymer at 24 and 48 h, respectively.

In summary, we have described phosphate ester and DNA cleaving properties of a metalated inorganic-organic hybrid polymer. The catalysis is heterogeneous in nature and the ease of separation of **CPPL–Zn** from the reaction medium and its ready recyclability are few attractive features of this system that can be further optimized for diverse chemico-biological applications.

Acknowledgements

We thank DST and CSIR, India for financial support. P.D., V.K., A.A., C.M. and S.G.S. thank CSIR for the award of Senior Research Fellowships.

References and notes

1. (a) Chandrasekhar, V.; Krishnan, V. *Adv. Inorg. Chem.* **2002**, *53*, 159. (b) Chandrasekhar, V.; Nagendran, S. *Chem. Soc. Rev.* **2001**, *30*, 193.
2. (a) Allcock, H. R.; Desorcie, J. L.; Riding, G. A. *Polyhedron* **1987**, *6*, 119. (b) Chandrasekhar, V.; Thomas, K. R. J. *J. Appl. Organomet. Chem.* **1993**, *7*, 1.
3. (a) Thomas, K. R. J.; Tharmaraj, P.; Chandrasekhar, V.; Bryan, C. D.; Cordes, A. W. *Inorg. Chem.* **1994**, *33*, 5382. (b) Thomas, K. R. J.; Tharmaraj, P.; Chandrasekhar, V.; Tiekink, E. R. T. *J. Chem. Soc., Dalton Trans.* **1994**, 1301. (c) Thomas, K. R. J.; Chandrasekhar, V.; Pal, P.; Scoot, S. R.; Hallford, R.; Cordes, A. W. *Inorg. Chem.* **1993**, *32*, 606. (d) Thomas, K. R. J.; Chandrasekhar, V.; Scott, S. R.; Hallford, R.; Cordes, A. W. *J. Chem. Soc., Dalton Trans.* **1993**, 2589.
4. Chandrasekhar, V.; Athimoolam, A.; Srivatsan, S. G.; Sundaram, P. S.; Verma, S.; Steiner, A.; Zacchini, S.; Butcher, R. J. *Inorg. Chem.* **2002**, *41*, 5162.
5. **Preparation of CPPL and CPPL–Zn**: **PPHVB** (1.20 g, 1.49 mmol) and 1, 4-divinylbenzene (80%, mixtures of isomers, 0.6 g) were polymerized together using AIBN (33 mg, 0.2 mmol) as the initiator in 1,2-dichloroethane. The cross-linked polymer, **CPPL** (1.53 g), was washed with

dichloromethane (3×20 mL), methanol (3×20 mL) and acetone (3×20 mL) and dried thoroughly under vacuum at 40 °C. Characterization data for **CPPL**: Anal. Found: C, 64.98; H, 6.2; N, 9.32. From the nitrogen analysis, it is calculated that every gram of the polymer contains 0.54 g of the cyclophosphazene moiety corresponding to 6.7×10^{-4} mol. **CPPL–Zn** was prepared by using anhydrous $ZnCl_2$ in dry methanol at room temperature for 36 h. The resulting metallated polymer was washed thoroughly with methanol (5×20 mL). From the AAS analysis, it was found that the amount of zinc present per gram polymer is equal to 11.5 mg.

6. Ochlal, E.-I. *J. Chem. Edu.* **1988**, *65*, 943.
7. Byun, Y.; Min, D.; Do, J.; Yun, H.; Do, Y. *Inorg. Chem.* **1996**, *35*, 3981.
8. **Kinetics**: The hydrolytic reactions were performed in duplicate in centrifuge tubes containing 3 mL of the substrate prepared in 0.01M *N*-ethylmorpholine buffer (pH 8.0) in 50% aqueous methanol at 32 °C. Methanol was used in the reaction solvent to ensure optimum wetting of the catalyst. The amount of polymer was 5 mg in the 3 mL of the buffer, corresponding to 0.29 mM of zinc, if the polymer was completely soluble in the buffer. Concentration of the hydrolytic product, *p*-nitrophenolate anion, was determined by measuring the absorbance of its time dependent release at 400 nm $\varepsilon_{400\ nm}$ ($16.5\times10^3\ M^{-1}.cm^{-1}$). The initial velocities were determined from concentration versus time plot and the pseudo-first-order rate constant was determined from $\ln(A_\infty/A_\infty-A_t)$ versus time plot. For K_m, V_{max}, concentrations of *h*NPP: 2.7–5.5 mM. For k_{obs}, concentration of *h*NPP was 5.5 mM.
9. Breslow, R.; Huang, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4080.
10. (a) Madhavaiah, C.; Srivatsan, S. G.; Verma, S. *Catal. Commun.* **2003**, *4*, 237. (b) Srivatsan, S. G.; Verma, S. *Chem. Eur. J.* **2002**, *8*, 5184. (c) Smith, K. L.; Tao, Z.-F.; Hashimoto, S.; Leitheiser, C. J.; Wu, X.; Hecht, S. M. *Org. Lett.* **2002**, *4*, 1079. (d) Negi, S.; Schneider, H.-J. *Tetrahedron Lett.* **2002**, *43*, 411. (e) Hartshorn, C. M.; Singh, A.; Chang, E. L. *J. Mat. Chem.* **2002**, *12*, 602. (f) Sreedhara, A.; Cowan, J. A. *J. Biol. Inorg. Chem.* **2001**, *6*, 337. (g) Gajda, T.; Düpre, Y.; Török, I.; Harmer, J.; Schweiger, A.; Sander, J.; Kuppert, D.; Hegetschweiler, K. *Inorg. Chem.* **2001**, *40*, 4918. (h) Yamaguchi, K.; Akagi, F.; Fujinami, S.; Suzuki, M.; Shionoya, M.; Suzuki, S. *Chem. Commun.* **2001**, 375. (i) Gast, F. U.; Franke, I.; Meiss, G.; Pingoud, A. *J. Biotech.* **2001**, *87*, 131. (j) Bodsgard, B. R.; Burstyn, J. N. *Chem. Commun.* **2001**, 647. (k) Kimura, E. *Curr. Opin. Chem. Biol.* **2000**, *4*, 207. (l) Komiyama, M.; Takeda, N.; Shigekawa, H. *Chem. Commun.* **1999**, 1443. (m) Krämer, R. *Coord. Chem. Rev.* **1999**, *82*, 243. (n) Bencini, A.; Berni, E.; Bianchi, A.; Fedi, V.; Giorgi, E.; Paoletti, P.; Valtancoli, B. *Inorg. Chem.* **1999**, *38*, 6323. (o) Chaudhuri, P.; Hess, M.; Müller, J.; Hildenbrand, K.; Bill, E.; Weyhermüller, T.; Wieghardt, K. *J. Am. Chem. Soc.* **1999**, *121*, 9599. (p) Trawick, B. N.; Daniher, A. T.; Bashkin, J. K. *Chem. Rev.* **1998**, *98*, 939. (q) Pogozelski, W. K.; Tullius, T. D. *Chem. Rev.* **1998**, *98*, 1089. (r) Burrows, C. J.; Muller, J. G. *Chem. Rev.* **1998**, *98*, 1109. (s) Bodsgard, B. R.; Burstyn, J. N. *Chem. Commun.* **2001**, 647. (t) Jeung, C. S.; Song, J. B.; Kim, Y. H.; Suh, J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 3061.
11. (a) Molenveld, P.; Engbersen, J. F. J.; Reinhoudt, D. N. *Chem. Soc. Rev.* **2000**, *29*, 75. (b) Deal, K. A.; Burstyn, J. N. *Inorg. Chem.* **1996**, *35*, 2792. (c) Kady, I. O.; Tan, B.; Ho, Z.; Scarborough, T. *J. Chem. Soc., Chem. Commun.* **1995**, 1137. (d) Raivji, G. H.; Milburn, R. M. *Inorg. Chim. Acta* **1988**, *150*, 227.
12. (a) Fritsky, I. O.; Ott, R.; Krämer, R. *Angew. Chem., Int. Ed. Engl.* **2000**, *39*, 3255. (b) Molenveld, P.; Engbersen, J. F. J.; Kooijman, H.; Spek, A. L.; Reinhoudt, D. N. *J. Am. Chem. Soc.* **1998**, *120*, 6726. (c) Suh, J.; Hong, S. H. *J. Am. Chem. Soc.* **1998**, *120*, 12545. (d) Morrow, J. R.; Buttery, L. A.; Berback, K. A. *Inorg. Chem.* **1992**, *31*, 16.
13. **DNA cleavage**: Cleavage reaction was performed by suspending 100 μg of **CPPL–Zn** (corresponding to 0.88 mM of zinc if the polymer was to be completely soluble in buffer) in sodium cacodylate buffer (8 mM, 20 μL, pH 7.5, 35 °C), containing pBR322 supercoiled plasmid DNA (10 ng/μL, New England Biolabs). 4 μL of methanol was used for polymer wetting. Individual reactions were quenched by adding gel loading buffer (5 μL) containing EDTA (100 mM) at regural time intervals. Reactions were loaded onto 0.7% agarose gel, containing ethidium bromide (1 μg/mL), and were electrophoresed for 1 h at constant current (80 mA). Gels were imaged on Bio-Rad Gel Documentation System 2000.
14. (a) Burrows, C. J.; Muller, J. G. *Chem. Rev.* **1998**, *98*, 1109. (b) Sigman, D. S.; Mazumder, A.; Perrin, D. M. *Chem. Rev.* **1993**, *93*, 2295. (c) Ueda, J.-I.; Takai, M.; Shimazu, Y.; Ozawa, T. *Arch. Biochem. Biophys.* **1998**, *357*, 231. (d) John, D. C. A.; Douglas, K. T. *Biochem. J.* **1993**, *289*, 463. (e) Reed, C. J.; Douglas, K. T. *Biochem. Biophys. Res. Commun.* **1989**, *162*, 1111.
15. **Lysozyme cleavage**: **CPPL–Zn** (200 μg) was suspended in 20 μL of 10 mM cacodylate buffer (pH 7.5) containing lysozyme (25 μM) at 35 °C. All reactions were quenched with 20 μL of sample buffer containing 20 μg of bromophenol blue and 20% of glycerol in 0.01 M of Tris–HCl buffer. Samples were loaded onto polyacrylamide gel (concentrating gel 4.5% and separating gel 12.5%) and electrophoresed for 8 h at constant voltage (80 V) in Tris–glycine buffer containing 0.1% sodium dodecyl sulphate. Gel was stained with Coomassie blue and destained prior to imaging on Bio-Rad Gel Doc 2000 system.