Nucleobase-Containing Metallated Polymeric Resins as Artificial Phosphodiesterases: Kinetics of Hydrolysis, pH Dependence, and Catalyst Recycling

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Abstract: Two metallated polymeric resins that contain the nucleobase adenine have been investigated for their ability to catalyze the hydrolysis of model phosphodiester substrates. These resins behave in a catalytic manner and display classical Michaelis–Menten kinetics. Consequently, parameters such as k_{obs}, K_m, V_{max}, and k_{cat} were determined for the two resins for both of the substrates. The most attractive feature of our nucleolytic system is its facile reusability that permits catalysis of multiple hydrolytic reactions, after resin recovery and a simple washing step, without significantly compromising the reaction rate. Involvement of a hydrolytic mechanism for phosphodiester cleavage is proposed on the basis of pH versus hydrolytic rate profiles for the two resins. We have also been able to demonstrate temperature-dependence, solvent effects, and inhibitory nature of vanadate ions on the observed rate of hydrolytic reaction aided by the resins. In conclusion, metallated nucleobase resins represent a new class of nucleolytic reagents and these systems have the potential to be further developed for multifarious applications in chemical biology.

Keywords: copper · hydrolysis · nucleobases · phosphodiesters · resins

Introduction

Enzyme-assisted phosphate ester hydrolysis is of considerable importance owing to its central role in key cellular processes.[1] Efforts to mimic this important biochemical reaction have lead to the exploration of numerous functional models that catalyze phosphate ester hydrolysis,[2] and many recent studies have sought understanding of the mechanism of rate enhancement by these reagents.[3] Most of the model systems for phosphate ester cleavage utilize inner or outer transition metal complexes[4] and the acceleration of hydrolytic reaction is either through metal-ion-assisted activation of water molecules or the generation of reactive radical species. In the former scenario, an increase in the observed rate of phosphate ester hydrolysis is generally attributed to the ability of ligand-bound metal ions to polarize water molecules; this makes them more acidic compared to free water molecules and thus facilitates attack at the phosphorus center.[5] In contrast, the oxidative free-radical mechanism may follow a Fenton-type reaction, for the generation of hydroxyl radicals, that leads to phosphate ester cleavage.[6]

We have recently reported preliminary observations of copper-metallated polymeric resin, containing adenine, which possess the ability to cleave phosphate monoesters with catalytic efficiency.[7] In these resins, an *N*-allyl group replaces the sugar residue at purine N9 position and nonhydrolyzable cross-linkers are used, instead of a phosphate backbone, to generate a molecular framework to hold multiple adenine residues in the polymeric matrix.[8] Metallation of adenine sites in the polymeric matrix is based on previous documented interactions of purines with metal ions such as Cu^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+}.[9] Copper metallation of the resin results in a constellation of multiple metal sites within the polymeric matrix. It is surmised that, akin to previously reported synthetic metallophosphatases, metal ions coordinated to adenine could also mitigate negative charges during the reaction, besides providing a metal-bound hydroxide for attacking phosphorus center[10] or a reactive oxygen species for phosphate ester cleavage.[6]

Both of the resinous catalysts were insoluble in all the common solvents studied and thus their application is heterogeneous in nature; this is in contrast to many synthetic dephosphorylation reagents reported to date. Remarkable rate enhancements have been observed with these resins, containing different cross-linkers, for the hydrolysis of two model phosphodiester substrates. Detailed kinetic analysis of resin-assisted cleavage, catalyst recycling, pH effect, inhibitory action of vanadate ions, and a proposed model for hydrolytic mechanism are presented in this report.

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Results and Discussion

Characterization of metallated nucleobase resins: Adenine-containing polymeric resins were prepared by AIBN-mediated free-radical polymerization (AIBN = azobisisobutyronitrile) of 9-allyladenine with a cross-linker, 1,4-divinylbenzene (DVB; **1**)[11] or ethyleneglycol dimethacrylate (EGDMA; **2**), in the presence of copper salts. The metallated resins were made rigid by taking higher equivalence of cross-linker so that they could withstand higher temperature and rugged reaction conditions. The extent of nucleobase incorporation was determined by elemental analysis, while the copper content in metallated resins was estimated by atom absorption spectrometry (AAS) or inductively coupled plasma (ICP) analysis.[12] It was interesting to observe a differential copper loading pattern for resins **1** and **2**. We suspect that it could be due to the presence of other functional groups in EGDMA in contrast the to DVB cross-linker. Metallated resins were further characterized by EPR spectroscopy and by preliminary magnetic susceptibility studies. Polymer **2** displayed a rhombic symmetry with $g_1 = 2.229$, $g_2 = 2.076$, and $g_3 = 2.031$ (Figure 1). Moreover, magnetic studies with metallated resins indicated paramagnetic nature of copper in resin **2**. Interestingly, metallated resin **1** was found to be diamagnetic and the presence of EPR peaks in its spectrum indicated the presence of trace amount of oxidized copper species within the polymeric matrix (data not shown). Both of the metallated resins were completely insoluble in common solvents and, thus, the catalysis of hydrolytic reactions was heterogeneous in nature.

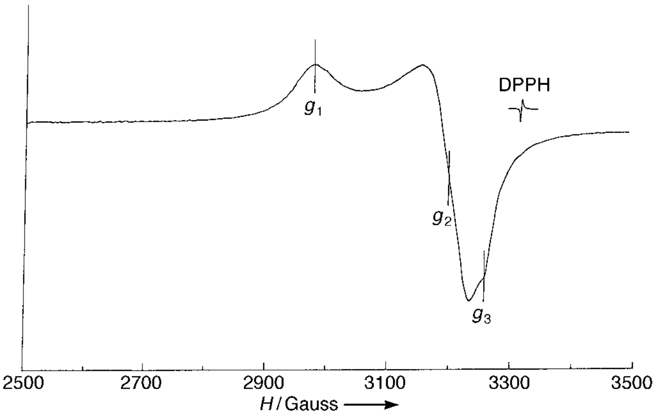

Figure 1. EPR spectrum of **2**.

Kinetic evaluation of phosphodiester hydrolysis and temperature dependence: We have extensively studied the hydrolysis of routinely used phosphodiester substrates such as bis(*p*-nitrophenyl) phosphate (bNPP) and 2-hydroxypropyl-*p*-nitrophenyl phosphate (hNPP), assisted by resins **1** and **2**. While bNPP acts as a phosphodiester substrate for an intermolecular hydrolytic reaction, hNPP acts as a RNA-like substrate, containing a phosphodiester linkage, for an intramolecular hydrolytic reaction, through possible activation of an internal nucleophile.

Significant rate enhancements were observed for the hydrolysis of both diester substrates by the two resins. Pseudo-first-order rate constants (k_{obs}) were determined from $\ln A_\infty / A_\infty - A_t$ versus time plots and for bNPP hydrolysis, it was found that resins **1** and **2** displayed a 4.81×10^5- and 2.05×10^6-fold rate enhancement, respectively, relative to the uncatalyzed reactions (Table 1).[4j] Two-million-fold acceleration of bNPP hydrolysis by resin **2** is at par with previously reported rate enhancements for this substrate by employing metallated ligands or by direct addition of metal ions.[4h,j] Similarly, k_{obs} values for hNPP hydrolysis for resins **1** and **2** were found to be $5.68 \times 10^{-3}\,min^{-1}$ and $7.19 \times 10^{-3}\,min^{-1}$, respectively, which correspond to 2.87×10^3- and 3.63×10^3-fold rate enhancement over the uncatalyzed reaction.[4k]

Table 1. Composition and pseudo-first-order rate constants for resins **1** and **2**.[a]

	9AA:cross-linker:$CuCl_2$	Substrate[b,c]	k_{obs} [min^{-1}]	k_{rel}
1	DVB (1:3:1)	bNPP	3.75×10^{-4}	4.81×10^5
		hNPP	5.68×10^{-3}	2.87×10^3
2	EGDMA (1:4:1)	bNPP	1.6×10^{-3}	2.05×10^6
		hNPP	7.19×10^{-3}	3.63×10^3

[a] All hydrolytic reactions were performed in duplicate in 3 mL of 0.01 M *N*-ethylmorpholine buffer in 50 % aqueous methanol (pH 8.0; 30 °C). The reference cell contained substrate without polymer to correct for background hydrolysis. [b] Weight of resins **1** and **2** were 1 mg mL^{-1}, correspondingly the concentration of bNPP was 7.0 and 10.0 mM, respectively.

Saturation kinetics of bNPP hydrolysis was observed using a Michaelis–Menten plot, in which substrate concentration was plotted against initial velocity of hydrolysis (Figure 2). Resins **1** and **2** were further subjected to detailed kinetic analysis in order to derive Michaelis–Menten parameters, K_m and V_{max}, and the turnover number, k_{cat}, for both of the substrates by using the Lineweaver–Burk plots (Table 2). For bNPP hydrolysis, with resin **1** as a catalyst, K_m, V_{max}, and k_{cat}, were found to be 37.2 mM, 6.76×10^{-5} mM min^{-1}, and $6.78 \times 10^{-5}\,min^{-1}$, respectively. While for resin **2**, corresponding values were 6.09 mM, 5.71×10^{-5} mM min^{-1}, and $1.66 \times 10^{-5}\,min^{-1}$ (Figure 3). These parameters were also obtained for the hydrolysis of RNA model substrate, hNPP. For resin **1**, the respective K_m, V_{max}, and k_{cat} values were found to be 1.32 mM, 2.35×10^{-4} mM min^{-1}, and $2.26 \times 10^{-4}\,min^{-1}$, while these values for resin **2** were 3.94 mM, 1.08×10^{-3} mM min^{-1}, and $9.23 \times 10^{-4}\,min^{-1}$, respectively (Figure 4). At this point, it

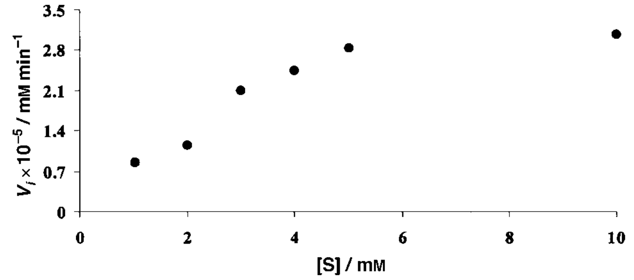

Figure 2. Michaelis–Menten plot for bNPP hydrolysis in which substrate concentration is plotted against initial velocity of hydrolysis.

Table 2. Michaelis-Menten kinetic parameters for resins **1** and **2**.[a]

	Substrate	K_m [mM]	V_{max} [mM min^{-1}]	k_{cat} [min^{-1}]
1[b]	bNPP	37.2	6.76×10^{-5}	6.78×10^{-5}
	hNPP	1.32	2.35×10^{-4}	2.26×10^{-4}
2[c]	bNPP	6.09	5.71×10^{-5}	1.66×10^{-5}
	hNPP	3.94	1.08×10^{-3}	9.23×10^{-4}

[a] All hydrolytic reactions were performed in duplicate in 3 mL of 0.01M *N*-ethylmorpholine buffer in 50% aqueous methanol (pH 8.0; 30°C). The reference cell contained substrate without polymer to correct for background hydrolysis. Polymer weights were 1 mgmL^{-1}, corresponding to 1.0 and 3.54 mM of copper, if the polymeric resins **1** and **2** were completely soluble in the buffer; [b] Concentrations of bNPP and hNPP for resin **1** were 3.0–7.0 mM and 0.25–1.0 mM, respectively; [c] Concentrations of bNPP and hNPP for resin **2** were 1.01–5.0 mM and 0.25–1.0 mM, respectively.

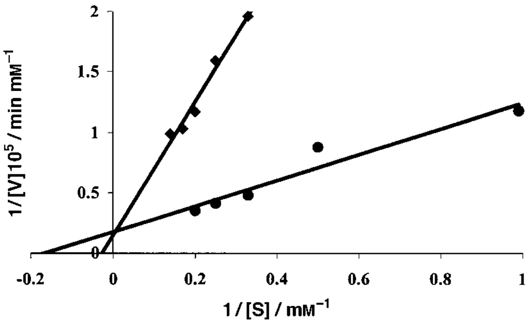

Figure 3. Lineweaver–Burk plots for bNPP hydrolysis catalysed by **1** (◆) and **2** (●).

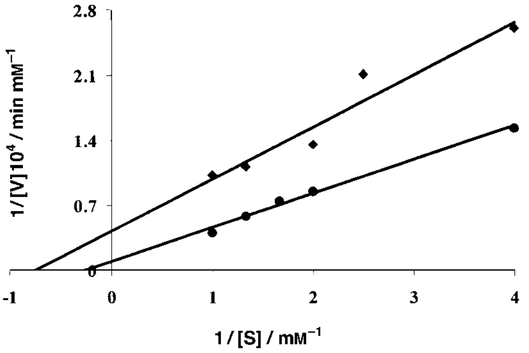

Figure 4. Lineweaver–Burk plots for hNPP hydrolysis catalysed by **1** (◆) and **2** (●).

is important to state that unmetallated cross-linked polymers were used as controls and under similar conditions they failed to promote phosphate ester hydrolysis over an extended period of time. Moreover, enhancement in the hydrolytic rates was not observed even if the substrates were incubated in buffer alone. All of the hydrolytic reactions were performed in duplicate and for over four half-lives of each substrate.

Table 3. Effect of temperature of resin **2** catalyzed bNPP hydrolysis.[a]

T [°C]	k_{obs} [min^{-1}]	Relative increase in k_{obs} for a 10°C rise
30	1.6×10^{-3}	–
40	3.16×10^{-3}	1.98
50	7.74×10^{-3}	2.45
60	11.01×10^{-3}	1.42

[a] All hydrolytic reactions were performed in duplicate in 3 mL of 0.01M *N*-ethylmorpholine buffer in 50% aqueous methanol at pH 8. 0. The reference cell contained substrate without polymer to correct for background hydrolysis. Weight of the resin **2** used was 1 mgmL^{-1} for each reaction. The substrate concentration was fixed at 10 mM.

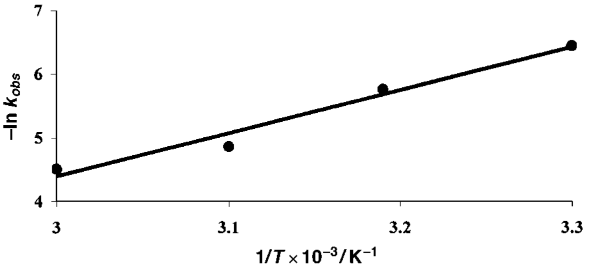

Figure 5. Plot of lnk_{obs} versus 1/T for **2** and bNPP.

The effect of temperature on the rate of bNPP hydrolysis catalyzed by resin **2** was evaluated. Pseudo-first-order rate constants were determined at four different temperatures (30–60°C) and the relative increase in k_{obs} values was nearly twofold for every 10°C rise in temperature (Table 3). Arrhenius parameters, E_a and A, were calculated from lnk_{obs} versus 1/T (Figure 5). The energy of activation was found to be 13.38 kcalmol^{-1}, while the frequency factor was 7.31×10^6 min^{-1}.Akin to most chemical reactions,[13] this data confirms temperature dependence of resin-catalyzed hydrolysis of a model phosphodiester substrate. It is important to note that prolonged use of resins at higher temperatures did not alter their catalytic efficiency, thus reinforcing our claim of the rugged nature of these resins.

pH dependence and solvent effects: Effect of pH on the activity of metallated resins was investigated by using bNPP as a substrate. Cleavage of bNPP, in the presence of resin **1** or **2**, was studied between pH range of 7.3–9.2, and pH versus k_{obs} plots were generated for both of the resins. It was interesting to note that a sigmoidal rate profile was obtained for both of the cases, suggesting a definite pH dependence on phosphodiester bond cleavage (Figures 6 and 7). Similar profiles have been previously reported in the literature for phosphate ester hydrolysis promoted by Cu^{2+},[14] Zn^{2+},[15] and Co^{3+}[16] complexes. As proposed in these reports, a sigmoidal rate profile is indicative of a hydrolytic mechanism, mediated by a metal-hydroxy or a metal-alkoxy intermediate, rather than a radical-induced cleavage. In this context, the ability of transition metal ions in lowering the pK_a value of water and attack of

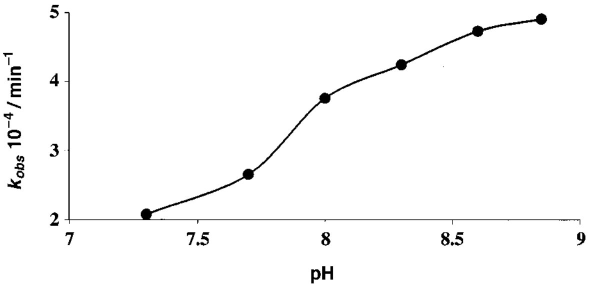

Figure 6. pH versus k_{obs} plot for the cleavage of bNPP in the presence of resin **1**.

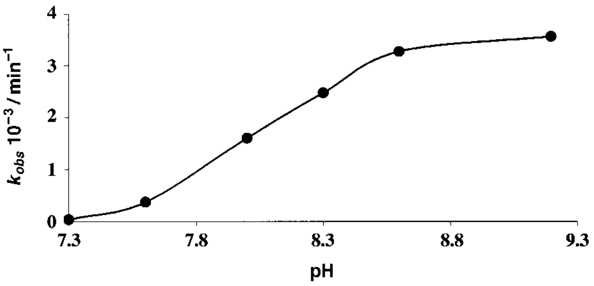

Figure 7. pH versus k_{obs} plot for the cleavage of bNPP in the presence of resin **2**.

water-bound hydroxyl at the phosphorus center in phosphate ester hydrolysis is already documented.[5] As all of our studies were performed in 50% aqueous methanol system, we also became interested in evaluating the effect of solvent on phosphodiester bond cleavage.

Preferential activation of attacking nucleophiles by metallated resins was determined by performing solvolytic reactions in water, ethylene glycol, and in 50% aqueous ethylene glycol with bNPP as a substrate. Ethylene glycol was used instead of methanol due to insufficient solubility of bNPP (disodium salt) in pure methanol. Pseudo-first-order rate constants were determined for the three systems (Table 4). Relative to the rate of solvolysis in pure ethylene glycol, a twofold increase in k_{obs} was found for 50% aqueous ethylene glycol system, while the rate enhancement was over fivefold if the reaction was performed in water alone. These observations clearly confirm efficiency of a metal-bound hydroxide ion in aiding bNPP hydrolysis, when compared to an intermolecular transesterification reaction facilitated by metal-bound alkoxy intermediate, as in the case of ethylene glycol (or methanol). However, alkoxide-ion-assisted transesterification is feasible and this approach has been briefly explored by Morrow et al. using a macrocyclic lanthanide complex that contained hydroxyethyl groups.[17] It will be of interest to perform detailed experiments to determine the course of intramolecular splicing of natural RNA substrates catalyzed by these resins, in order to decipher the role of metal ions present in our resins for the activation of the 2′-OH group present in RNA.

Table 4. Effect of solvent on resin **2** catalyzed bNPP solvolysis.[a]

Solvent	k_{obs} [min^{-1}]	K_{rel}[b]
ethylene glycol (100%)	0.40×10^{-3}	1.0
water/ethylene glycol (1:1)	0.80×10^{-3}	2.0
water (100%)	2.17×10^{-3}	5.4

[a] All hydrolytic reactions were performed in duplicate in 3 mL of 0.01M *N*-ethylmorpholine buffer in respective solvents (pH 8.0, 30°C). The reference cell contained substrate without polymer to correct for background hydrolysis. Weight of the resin **2** used was 1 mg mL^{-1} for each reaction. The substrate concentration was fixed at 10mM. [b] Relative rates are reported with respect to k_{obs} obtained for pure ethylene glycol.

Recycling experiments: Polymer-based reagents for phosphate ester hydrolysis offer an attractive paradigm for catalyst design.[18] However, the most unique feature of the resins described in this report is their facile recovery and reusability. Due to the insolubility of these polymeric matrices in common solvents and, therefore, the heterogeneous nature of catalytic reaction, it is quite simple to recover the resins by centrifugation after the completion of a hydrolytic reaction. In a typical procedure, the recovered resin is washed by 50% aqueous methanol (6 × 5 mL), methanol (3 × 5 mL), and acetone (2 × 5 mL), and air-dried. We found that recycled resins **1** and **2** could continuously catalyze several hydrolytic reactions for different substrate (bNPP and hNPP) concentrations and, more importantly, it was found that the initial velocities obtained for recycling experiments were similar to those observed when fresh catalysts were used (Table 5). These experiments demonstrate the rugged and truly catalytic nature of the resins that can be exploited for accelerating several hydrolytic reactions without losing catalytic efficiency. After three cycles of hydrolytic reactions, the copper content in resin **2** was determined by using AAS. No significant loss in the concentration of copper from the polymeric matrix was observed for the recycled resin, and indication of its robust molecular structure (data not shown).

Table 5. Recycling kinetics with resins **1** and **2**.[a]

	Substrate	Substrate concentration [mM]	V_i [mM min^{-1}] fresh polymer	recovered poylmer
1	bNPP	3.0	5.09×10^{-6}	7.93×10^{-6}
		4.0	6.28×10^{-6}	6.66×10^{-6}
		5.0	8.57×10^{-6}	8.94×10^{-6}
2	bNPP	2.0	1.14×10^{-5}	1.06×10^{-5}
		3.0	2.09×10^{-5}	1.78×10^{-5}
		4.0	2.43×10^{-5}	2.3×10^{-5}
2	hNPP	0.25	0.65×10^{-4}	0.63×10^{-4}
		0.5	1.17×10^{-4}	1.23×10^{-4}
		0.75	1.74×10^{-4}	1.88×10^{-4}

[a] All hydrolytic reactions were performed in duplicate in 3 mL of 0.01M *N*-ethylmorpholine buffer in 50% aqueous methanol (pH 8.0; 30°C). The reference cell contained substrate without polymer to correct for background hydrolysis. Weight of the resins used were 1 mg mL^{-1} for each reaction.

Inhibition of resin-assisted hydrolysis by vanadate ions: The vanadate ion is a potent inhibitor of phosphatases and related enzymes due to its ability to mimic the transition state of phosphate ester hydrolysis.[19] In this vein, we decided to evaluate the effect of vanadate on the catalytic activity of our polymeric resins. The pseudo-first-order rate constant was determined for bNPP hydrolysis catalyzed by resin **2**, in absence and presence of 0.4mM ammonium vanadate at pH 8.3, under standard buffer conditions. A comparison of k_{obs} values obtained for resin **2** in the absence (2.47×10^{-3} min^{-1}) and in the presence of vanadate anion (8.35×10^{-4} min^{-1}) revealed a threefold decrease in the observed reaction rate. Although the reduction in k_{obs} is not dramatic, the observations may be attributed to the mimicry of the transition state for phosphate ester hydrolysis, as mentioned before. In another scenario, Cu^{2+} ions present in the resin might get complexed to vanadate ions, thereby rendering catalyst ineffective toward phosphate ester hydrolysis. We are currently in the process of determining the basis of vanadate-induced inhibition of hydrolytic reaction catalyzed by these resins for devising a model for the inhibitory effect observed.

Conclusion

Synthesis and catalytic activity of metallated adenine-containing resins have been described. Remarkable rate enhancements were observed for the hydrolysis of two model phosphodiester substrates, and the fact that these resins are also amenable to facile recovery and recycling adds to their catalytic potential and broad utility. A possible model of substrate binding to metallated adenine nucleobases within the polymeric matrix, through hydrogen bonding, is shown in Figure 8. It resembles other proposals for metal ion-assisted hydrolysis of phosphodiester substrates.[18a]

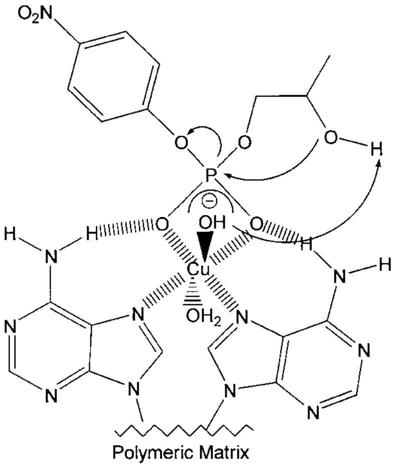

Figure 8. A possible model of substrate binding to metallated adenine nucleobases within the polymeric matrix.

We hope that this new heterogeneous catalytic system will lead to the development of better nucleolytic reagents for chemistry and biology. We intend to further probe the mechanism of catalytic action of these resins and apply this system to other substrates like cyclic nucleotides, dinucleotides, and phosphoanhydrides. Moreover, we are also devising strategies to engineer specificity in polymeric resins for sequence-selective and targeted cleavage of natural substrates, such as dsDNA and ssRNA.

Experimental Section

Instrumentation: The amount of copper in the metallated-nucleobase polymer was determined by ICP analysis on Integra XL AAS spectrometer (GBC) at the FEAT Laboratory, IIT-Kanpur. EPR spectra were recorded on a Varian 109E Line Century Series, X-band spectrometer, at liquid nitrogen temperature. Elemental analyses were performed at the Regional Sophisticated Instrumentation Centre, Lucknow. Reaction kinetics was performed on a Shimadzu UV-160, UV/Vis spectrophotometer.

Chemicals and reagents: Ethyleneglycol dimethacrylate (EGDMA) was purchased from Fluka (Switzerland) and washed with 10% aqueous NaOH prior to use. AIBN (Ajax Chemicals, India) was recrystallized from methanol. $CuCl_2 \cdot 2H_2O$ (Merck, India) and pNPP (SRL, Mumbai) were used as supplied. The sodium salt of bNPP (Fluka, Switzerland) was prepared from the commercial sample and used for kinetic assays. *N*-Ethylmorpholine, methanol (spectroscopic grade), and ethylene glycol were from S.D. Fine Chemicals (India) and were distilled prior to use. Triply distilled water was used in all assays.

Syntheses:

Cross-polymer of 9-allyladenine (9-AA) and EGDMA: AIBN-initiated free-radical polymerization was employed for the synthesis of metallated nucleobase polymers. 9-AA (0.1 g, 0.57 mmol, 1 equiv), EGDMA (0.4 mL, 2.1 mmol, 3.7 eq), and AIBN (0.1 g) were dissolved in chloroform/methanol (5:3, 12 mL), and the reaction mixture was purged with oxygen-free N_2 gas for 45 min. Polymerization was performed at 55–60 °C for 15 h. The white glassy solid formed was filtered, washed with methanol (5 × 20 mL), chloroform (2 × 15 mL), and benzene (2 × 15 mL), and air-dried to afford 0.4 g of the cross-linked polymer. Elemental analysis found (%): C 53.5, H 6.47, N 0.92. From nitrogen analysis, it was concluded that every gram of resin contains 22.9 mg of 9-AA; this corresponds to a value of 0.13 mmol.

*Metallated cross-polymer of 9-AA and EGDMA (**2**)*: 9-AA (0.4 g, 2.29 mmol, 1.0 eq), EGDMA (1.73 mL, 9.16 mmol, 4.0 equiv), and $CuCl_2 \cdot 2H_2O$ (0.391 g, 2.29 mmol, 1 eq), were dissolved in chloroform/methanol (5:3, 44 mL), and the reaction mixture was purged with oxygen-free N_2 gas for 45 min. Polymerization was performed at 55–60 °C for 30 h. The residue was filtered, washed with methanol (5 × 20 mL), chloroform (2 × 25 mL), and benzene (2 × 25 mL), and air-dried. The metallated polymer was obtained as a green amorphous powder (0.215 g) and was found to be insoluble in common organic solvents and water.

*Characterization of polymer **2***: Elemental analysis found (%): C 29.06, H 2.89, N 22.65. From nitrogen analysis, it was concluded that every gram of resin contains 566 mg of 9-AA; this corresponds to a value of 3.2 mmol. EPR spectroscopy: The EPR spectrum of polymer **2**, at liquid nitrogen temperature (77 K), displayed a rhombic symmetry with $g_1 = 2.229$; $g_2 = 2.076$, and $g_3 = 2.031$. Atomic absorption spectroscopy: The amount of copper in metallated resin **2** was estimated by AAS and was found to be 225 mg of copper per gram of polymer.

Kinetics of hydrolysis: All hydrolytic reactions were performed in duplicate in centrifuge tubes thermostatted at 30 °C. The assay mixture contained 3 mL of substrate solution of appropriate concentration prepared in 0.01 M *N*-ethylmorpholine buffer (pH 8.0) in 50% aqueous methanol. The amount of polymer was $1\,mg\,mL^{-1}$ of buffered substrate solution and the concentration of the catalyst corresponded to the amount of copper present in the polymeric matrix. Initial velocities were determined as a function of time-dependent release of *p*-nitrophenolate anion ($\varepsilon_{400} = 1.65 \times 10^4\,M^{-1}\,cm^{-1}$). Michaelis–Menten parameters were calculated from corresponding Lineweaver–Burk plots. The pseudo-first-order rate constants were derived from $\ln A_\infty / A_\infty - A_t$ versus time plots. All of the hydrolytic reactions were performed at least over four half-lives of each substrate.

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